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Hemorrhagic shock resulting from injury is the most common cause of traumatic death in both civilian and military settings. Worldwide, over 5 million people died from trauma-related injury in 2000 with up to 64% of those fatalities having suffered a coinciding brain injury. For nearly a century, researchers have investigated resuscitative strategies to better the outcomes of traumatic casualty treatments. The initial measures in management, hemostasis and volume restoration are vital but too often prove unsuccessful in improving morbidity and mortality. As a result, our team investigated the hypothesis that diazoxide (DZ), a mitochondrial KATP channel opener, could be used during resuscitation to induce a phenomenon that is called an ?ischemic postconditioning effect? in a laboratory animal model of hemorrhagic shock leading to systemic hypotension and unilateral cerebral ischemia. Male Sprague-Dawley rats underwent a 40% plasma volume hemorrhage with resuscitation one hour later. DZ was administered at one of three time points: 20 minutes before the onset of resuscitation, at the time of resuscitation, or 20 minutes after the initiation of resuscitation.

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ABSTRACT

THE POSTCONDITIONING EFFECTS OF DIAZOXIDE IN THE BRAIN FOLLOWING HEMORRHAGIC SHOCK AND CEREBRAL HYPOPERFUSION

Major Michael W. Bentley

Thesis directed by Joseph T. McCabe, Ph.D., Vice-Chair of Department of Anatomy, Physiology & Genetics; and Professor of Molecular and Cellular Biology, and Neuroscience.

Abstract

Hemorrhagic shock resulting from injury is the most common cause of traumatic death in both civilian and military settings. Worldwide, over 5 million people died from trauma-related injury in 2000 with up to 64% of those fatalities having suffered a coinciding brain injury. For nearly a century, researchers have investigated resuscitative strategies to better the outcomes of traumatic casualty treatments. The initial measures in management, hemostasis and volume restoration are vital but too often prove unsuccessful in improving morbidity and mortality. As a result, our team investigated the hypothesis that diazoxide (DZ), a mitochondrial K_{ATP} channel opener, could be used during resuscitation to induce a phenomenon that is called an “ischemic postconditioning effect” in a laboratory animal model of hemorrhagic shock leading to systemic hypotension and

unilateral cerebral ischemia. Male Sprague-Dawley rats underwent a 40% plasma volume hemorrhage with resuscitation one hour later. DZ was administered at one of three time points: 20 minutes before the onset of resuscitation, at the time of resuscitation, or 20 minutes after the initiation of resuscitation.

Western blotting and immunohistochemistry results showed when DZ was administered intravenously 20 minutes prior to resuscitative efforts following hemorrhagic shock, that the cytoprotective heat shock proteins (HSP) 25 and HSP70 exhibited marked upregulation in ischemic areas. Further immunohistochemistry analysis demonstrated that DZ when given simultaneous with resuscitation decreased the apoptotic cleaved caspase-3 protein within the cerebral cortex and hippocampus. DZ given at the time of resuscitation in the initial resuscitation volume significantly reduced caspase-3 activity bilaterally in ischemic and hypotensive regions of the hippocampus and the perirhinal cortex following hemorrhagic shock. Taken together, these results suggest DZ has a postconditioning effect that may confer brain protection and limit apoptosis following hemorrhagic shock and cerebral hypoperfusion by increasing heat shock proteins and decreasing caspase-3 activity. These effects may be independent of each other depending on the time point at which DZ is administered.

THE POSTCONDITIONING EFFECTS OF DIAZOXIDE IN THE BRAIN FOLLOWING HEMORRHAGIC SHOCK AND CEREBRAL HYPOPERFUSION

by

MAJ Michael W. Bentley

US Army Nurse Corps

Doctoral Dissertation submitted to the faculty of the Graduate Program in Neuroscience of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 2009

DEDICATION

I dedicate this work to my beautiful wife, Elana.

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LIST OF ABBREVIATIONS

ABG	arterial blood gas
ABP	arterial blood pressure
ADP	adenosine diphosphate
AIF	apoptotic inducing factor
AKT	protein kinase B
AMP	adenosine monophosphate
APAF-1	apoptotic protease activating factor-1
APO-2L	Apo2 ligand
ATP	adenosine triphosphate
BV	blood volume
^{o}C	Celsius
Ca^{2+}	calcium ion
CBF	cerebral blood flow
cc	cubic centimeter
CC3	cleaved caspase-3
CO_2	carbon dioxide

ddH_2O	double distilled water
DR4	death receptor 4
DR5	death receptor 5
DZ	diazoxide
DZ40	diazoxide given 40 minutes after hemorrhagic shock
DZ60	diazoxide given 60 minutes after hemorrhagic shock
DZ80	diazoxide given 80 minutes after hemorrhagic shock
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal related kinase
FJC	fluorojade C
FTMRV	first ten minutes resuscitation volume
g	gram
GFAP	glial fibrillary acidic protein
GSKβ	glycogen synthase kinase beta
H^+	hydrogen ion

H_2O	water
H_2O_2	hydrogen peroxide
HCO_3^-	bicarbonate ion
HRP	horse radish peroxidase
HSCH	hemorrhagic shock cerebral hypoperfusion
HSP	heat shock protein
HSP25	heat shock protein 25
HSP27	heat shock protein 27
HSP70	heat shock protein 70
IED	improvised explosive device
IM	intramuscular
IV	intravenous
K^+	potassium ion
K_{ATP}	potassium ATP sensitive channel
Kg	kilogram
Kir	potassium inward rectifying channel
LAD	left anterior descending artery

LCA	left carotid artery
MAC	minimum alveolar concentration
MAPK	mitogen activated protein kinase
MEK	extracellular signal-regulated protein kinase
min	minute
mK_{ATP}	mitochondrial potassium ATP sensitive channel
ml	milliliter
mm	millimeter
mM	micromolar
mmHg	millimeter of mercury
mPTP	mitochondrial permeability transition pore
Na	sodium
NAD^+	nicotinamide adenine dinucleotide ion
NADH	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NBD	nucleotide binding domain
nm	nanometer

NS	normal saline
O_2	oxygen
PBS	phosphate buffered saline
pH	logarithmic hydrogen ion concentration
Pi	inorganic phosphate
PI3K	phosphoinositide-3 kinase
PKC	protein kinase C
PKG	protein kinase G
RCA	right carotid artery
RISK	reperfusion ischemia survival kinase
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SUR	sulfonyurea receptor
TRAIL	TNF-related apoptosis-inducing ligand
UCAO	unilateral carotid artery occlusion
UCAO DZ	unilateral carotid artery occlusion receiving diazoxide

UCAO VEH	unilateral carotid artery occlusion receiving vehicle
USUHS	Uniformed Services University of the Health Sciences

Chapter 1

INTRODUCTION

Vale's 1904 review [1] attributes Henri Francois LeDran's 2nd French edition of *Treatise of Reflections Drawn from Experience with Gunshot Wounds* as the first clinical reference to shock [2]. Benjamin Travers is often referred to as defining shock in its present form as "a species of functional concussion by which the influence of the brain over the organ of circulation is deranged or suspended" [3]. Samuel Gross elegiacally defined battlefield shock during the first World War as "the rude unhinging of the machinery of life" [4]. But simply defined, shock is the period of transition from illness or injury to death resulting from the inability to transport or utilize oxygen at the cellular level.

It is estimated that over 5 million people died worldwide in 2000 from trauma related injuries [5]. In the United States, traumatic injury affects an estimated 10% of the population each year. Trauma is the fifth leading cause of death with 40% of those deaths a result of hemorrhagic shock. Fifty percent of trauma deaths involve persons between 15 and 44 years of age and 30-40% of those victims perish [6-9]. An estimated 117 billion dollars or 10% of the United States' total medical expenditures went for the treatment of trauma-related injuries in 2000 [8]. Moreover, between 2003 to 2006, 3.3 billion dollars in supplemental funding was appropriated for medical support of the Global War on Terror [10] To these horrendous statistics, the modern world has seen the increase of terroristic use of improvised explosive devices (IEDs), which can

result in the proliferation of high-kinetic energy tissue and organ penetration and severe vascular injuries [11]. Global terroristic activities and continued military operations are escalating worldwide, placing civilian and military populations at continued risk for traumatic injury.

Battle-inflicted injury and accidental trauma have long been associated with shock. Aside from the instantaneous lethality of injury, the most significant cause of death in the modern era following traumatic injury in civilian and military settings is hemorrhagic shock [9, 12]. In combat, hemorrhagic shock is the leading cause of death and responsible for 50% of combat fatalities with 64% of those casualties suffering a coinciding brain injury [13, 14].

Life-threatening injury can result in massive blood loss in a very short period of time. Hemorrhage produces a decreased supply of oxygen to all vital organs plunging the body into a global ischemic condition. This results in the activation of several biochemical processes that lead to cellular apoptosis, necrosis, organ failure, and ultimately severe impairment or death. For the last century, medical personnel have explored the utility of a range of resuscitative strategies that might improve the survival of trauma victims. Initial actions are deemed vital for the survival of injured persons—blood loss must be controlled and intravascular volume re-established. Too often, however, the resuscitative measures implemented throughout the echelons of care are unsuccessful. As well, resuscitation itself is not benign. The restoration of blood volume and tissue reoxygenation following hemorrhage initiates a secondary reperfusion injury [15].

Fluid considerations for trauma resuscitation are still the subject of considerable debate. Researchers continue to vary conditions concerning the volume, timing, and type of resuscitative fluid attempting to determine whether resuscitative therapies subsequent to hemorrhage that optimize organ survival can be developed [16-19]. One thing is clear: resuscitative strategies used subsequent to trauma and hemorrhagic shock that confer organ protection by encouraging a prosurvival phenotype are likely to be exciting opportunities for improving trauma care. Investigations are beginning to center on resuscitative fluids modified with pharmaceutical agents specifically chosen to augment fundamental survival pathways [20].

Cerebral Injury and Hemorrhagic Shock

As previously stated, 64% of fatalities resulting from hemorrhagic shock have a coinciding brain injury. This results from the impact of the traumatic event and/or from hypoxia secondary to the hemorrhage. Cerebral blood flow (CBF) and metabolism have been extensively studied. As reviewed by Vavilala and colleagues [21], the brain is dependent upon a constant supply of oxygen and glucose to conduct aerobic metabolism and oxidative phosphorylation. Up to 92% of the ATP generated in the brain comes from the aerobic metabolism of glucose. The brain has a limited ability to store glucose and thus is dependent on a constant blood flow for substrate delivery. Without a constant blood supply, ATP levels can reach zero within seven minutes after the cessation of blood flow. The normal cerebral blood flow is on average 50ml/100g of tissue/minute. Once

the mean arterial pressure falls below 60 mmHg, CBF begins to markedly decline. It is commonly quoted in textbooks that neuronal death will occur once the CBF falls to 6-12 ml/100g of tissue/min. One meta-analysis suggests that this threshold for neuronal death may be 4.8 to 8.4 ml/100 g of tissue/min [22]. In terms of mean arterial pressure, the critical pressure in animals for neuronal damage occurs when mean arterial pressure levels fall to 25-35 mmHg [23]. While it is debatable, the point is clear that at some critical threshold, a loss of cerebral perfusion pressure results in brain injury. Also, when approaching a 40% blood loss, oxygen consumption has been found to increase 160% of control values [24]. This loss of blood supply coupled with increased oxygen consumption makes the brain susceptible to ischemic injury following hemorrhagic shock. In humans that suffered only a hemorrhagic injury with no evidence of direct brain trauma, damage was found in the bilateral frontal and parietal-occipital cerebral cortices, the left middle cerebral peduncle, and bilateral cerebellar hemispheres. It was additionally noted that a decrease in cerebral perfusion pressure was thought to occur uniformly throughout all vascular territories in the brain based upon magnetic resonance angiography [25].

Pathophysiology of Hemorrhagic Shock

Shock is a result of the inability to transport oxygen to the mitochondria and/or for the mitochondria to carry out aerobic metabolism. With hemorrhagic shock, there is an abrupt loss of blood volume and cardiac output. This drop in cardiac output markedly slows the delivery of oxygen to organs as total oxygen

delivery (DO_2) is a function of the cardiac output (Q) and arterial oxygen content (CaO_2); $DO_2 = Q + CaO_2$ [26-28]. The body responds by releasing catecholamines and arginine vasopressin while increasing the activity of the renin-angiotensin system [29-31]. This raises peripheral vascular resistance and redistributes blood flow to the vital organs for the maintenance of adequate perfusion pressure and cellular delivery of oxygen. When cardiac output is no longer capable of oxygen delivery, metabolic requirements for oxygen will exceed available supply and an oxygen debt is created.

Intracellular pathology is a result of mitochondrial dysfunction. The Krebs cycle, the Chemiosmotic Theory, and ATP synthesis have been well described for decades [32-34]. Oxygen serves as the terminal electron acceptor at Complex IV in the electron transport chain. In the absence of oxygen, mitochondrial respiration slows [35] and NAD^+ cannot be reduced to NADH within the electron transport chain. Ultimately, pyruvate cannot be converted into Acetyl CoA for entry into the Krebs cycle. As a result, pyruvate acted upon by the enzyme Lactate Dehydrogenase within the cytosol and mitochondria produces lactate and NAD^+ [36]. Anaerobic conversion of lactate into ATP is highly inefficient as only 2 moles of ATP can be produced per mole of glucose, while under aerobic conditions one mole of glucose can produce 30 – 38 moles of ATP. This 5-6% efficiency of anaerobic metabolism for ATP production will eventually become lethal. Over time, ATP requirements for cellular homeostasis will exceed production and ATP levels will reach zero. As the duration of ischemia continues, energy dependent ionic pumps fail causing a rise of

intracellular ions. This increases intracellular osmotic pressure and cellular swelling. Eventually, cell membrane integrity is lost.

While anaerobic metabolism produces lactate, it is not responsible for the increase in hydrogen ion concentration and hence acidosis. An increase in lactate is only indicative of poor oxygen utilization by cells. The hydrolysis of ATP yields ADP, Pi and a hydrogen ion. During hypoxia, the hydrolysis of ATP exceeds its production yielding a net rise in hydrogen ion and acidosis ($ATP + H_2O \rightarrow ADP + Pi + H^+$ [37, 38]). Overall, there is a drop in the ATP/ADP ratio, a build-up of lactic acid, and a drop in intracellular pH. To correct for the acidosis, the cell utilizes the Na^+/H^+ antiporter, but this loads the cell with Na^+. Because of the drop in ATP, Na^+ cannot be pumped out. As a result, the cell loads with Ca^{2+} through the reversal of the Na^+/Ca^{2+} antiporter augmenting Ca^{2+} entry via uniport Ca^{2+} channel activity [39]. Ultimately, mitochondrial energetic dysfunction leads to a progressive failure of cellular homeostasis as the levels of Na^+, Ca^{2+}, and H^+ rise while the level of ATP plummets.

A rise in base deficit defines a decrease in serum bicarbonate. Bicarbonate ion reacts with hydrogen to form water and carbon dioxide ($HCO_3^- + H^+ \rightarrow H_2O + CO_2$). Under hypoxic conditions, hydrogen concentrations rise and the resulting acidosis is buffered in part by bicarbonate ion to yield water and carbon dioxide. When buffering occurs, the amount of bicarbonate ion (or base) falls and a base “deficit” is created. Taken together, the rise in plasma lactate levels, hydrogen ion concentration, and base deficit are all consequences of

mitochondrial dysfunction, and serve as clinically useful endpoints for assessing physiological status and the effectiveness of resuscitation [40-43].

Treatment of Hemorrhagic Shock

According to the 7th Edition of the Advanced Trauma Life Support for Doctors, warmed isotonic electrolyte solutions are used for initial resuscitation following shock with up to 2 liters given as a fluid bolus as rapidly as possible. This is followed by the replacement of each ml of blood lost with a crystalloid solution until the total amount of crystalloid administered is three times that of the total blood loss [44]. On the battlefield, it is recommended that initial fluid resuscitation of the hemorrhaging battlefield casualty should be a 250 ml bolus of 7.5% hypertonic saline delivered by a rapid-infusion system [12]. Recently, it has been suggested that upon arrival to an emergency department, hemorrhaging patients should be resuscitated in two phases. Initially, resuscitative measures should be limited to maintain a systolic blood pressure at 90 mmHg to prevent the dislodging of recently clotted vessels. Secondly, intravascular volume should be restored using thawed plasma as the primary resuscitation fluid in a 1:1 or 1:2 ratio with packed red blood cells [45]. However, these protocols do not address any maneuvers using fluids modified with pharmaceutical agents that activate cellular survival pathways which may reduce organ primary hypoxic injury and limit secondary reperfusion injury.

Reperfusion Injury

The restoration of blood flow is imperative to prevent irreversible necrotic or apoptotic injury of cells. However, abrupt reperfusion may actually expand irreversible injury by killing compromised but viable cells. In general, abrupt reperfusion 1) precipitously increases oxygen tensions promoting the *rapid and persistent* generation of reactive oxygen species (ROS), 2) activates the Na^+/H^+ exchanger and the Na^+/Ca^{2+} exchanger, 3) increases cytosolic and mitochondrial Ca^{2+} levels, 5) opens the mitochondrial Permeability Transition Pore (mPTP), 6) creates broad endothelial dysfunction & general cell lysis and 7) generates microvascular flow defects (no-reflow phenomenon) ([46]; Figure 1). Clinical profiles of the manifestations associated with reperfusion injury are described by Eltzschig and Collard [47]. “Stunning” takes place in myocardial tissue. ROS toxicity occurs; there is a decline in the re-synthesis of ATP, and an imbalance of cellular calcium uptake versus release. In patients undergoing revascularization procedures, arrhythmias are common. Arrhythmias correlate with an increase in the morbidity and mortality associated with stroke, head injury, circulatory arrest, and aneurysm repair. With gastrointestinal pathologies, abrupt reperfusion decreases intestinal barrier function. Finally, abrupt reperfusion increases the incidence of multi-organ systems failure in the critically ill patient. In total, clinical profiles establish that reperfusion can amplify organ injury and increase morbidity and mortality.

Postconditioning

There are clear needs to further improve hemorrhagic shock treatment protocols and to minimize tissue damage resulting from reperfusion injury. One promising approach for improving resuscitation therapies may be derived from the phenomenon call *postconditioning*, in which protective measures are employed following an injury. The roots of postconditioning stem from the work of Okamoto and colleagues [48]. Okamoto's group established post-ischemic damage could be limited by the use of timely low-pressure reperfusion. Following a period of ischemia, dog hearts were reperfused either with the sudden release of a coronary occlusion, or by low-pressure (40 to 50 mm Hg) coronary reperfusion with normal blood for 20 minutes before completely removing the coronary occlusion. This maneuver focused on the initial stage of reperfusion and established the basis for novel postconditioning approaches to resuscitation.

In 2003, Zhao and colleagues [49] first used the term postconditioning. They found in a model of occlusive hypoxia in dogs that short, repeated periods of arterial occlusion and release of previously occluded coronary arteries (three occlusions of 30 seconds each) just prior to reperfusion reduced infarct area by 44%. This was an example of a mechanical postconditioning intervention and implied that the first minute of reperfusion is critical in thwarting cellular demise. Years earlier, Mizumura and colleagues [50], first demonstrated pharmacological postconditioning. Mizumura's group used the potassium ATP-sensitive (K_{ATP})

channel opener, bimakalin, in dogs. Bimakalin markedly reduced cardiac infarct size when given 10 minutes before and during the 60-minute coronary reperfusion period following a set time of occlusive hypoxia. Collectively these initial findings firmly established the concept of postconditioning and emphasized a critical factor that altering the initial moments of reperfusion was beneficial. These findings were further validated by Kin and colleagues [51]. Kin and colleagues stated that the first minute of reperfusion in the rat was crucial for postconditioning. Three cycles of 10 second coronary occlusion and 10 second coronary release preceding a full coronary occlusion release decreased cardiac infarct size by 23%.

Researchers are keenly interested in delineating the specific signal pathways that mediate postconditioning. Recent findings from the employment of mechanical and pharmacological postconditioning suggest the activation of mitochondrial potassium ATP-sensitive (mK_{ATP}) channels by agents such a diazoxide (DZ). As discussed below, activation of this channel appears to initiate a series of events that close the mitochondrial permeability transition pore (mPTP) and converge onto the Reperfusion Injury Survival Kinase (RISK) Pathway.

Diazoxide

In trauma treatment settings, an agonist employed during resuscitation that acts upon mK_{ATP} channels could serve as a useful postconditioning trigger. DZ (3-methyl-7-chloro-1,2,4-benzo-thiadiazine,-1,1-dioxide) is a nondiuretic

benzothiadiazine with antihypertensive properties, which has been used in the treatment of hypertension since the early 1960s [52]. It is an odorless, white crystalline substance with a molecular weight of 230.7 grams with a melting point of 330^{o}C [53]. It has an onset of action of 1-2 minutes when given intravenously and a half-life of 17-35 hours for a single dose but the hypotensive actions last for two hours. In humans, a dose of 7.5 mg/kg is required to elicit hypotensive effects but infusions of 3.5 mg/min do not create hypotension [54, 55]. DZ is a mK_{ATP} and plasma membrane K_{ATP} channel opener with a 2000-fold higher affinity for the mK_{ATP} channel than for the plasma membrane KATP channel [56]. DZ also decreases cellular respiration through the inhibition of cytochrome II and attenuates ROS. Those actions are dependent upon the energy state of the cell [57, 58].

DZ appears to induce postconditioning effects by acting upon mK_{ATP} channels during ischemia. Activation during ischemia increases mitochondrial matrix K^+ and matrix volume as obligate water follows K^+. Overall during ischemia, matrix pH drops but matrix pH maintains a higher value with the entry of K^+ as a result of the activity of the K^+/H^+ exchanger (K^+ out / H^+ in). The K+/H+ exchanger activity decreases proton gradients that drive ATP production and in turn decreases electron motive force. A slower electron motive force allows superoxide ion production by Complex I. Superoxide dismutase generates signaling ROS, which catalyzes the activation of prosurvival kinases. These kinases can close mPTP and cyclically enhance the opening of the mK_{ATP}

channel (Figure 2; [56, 59-62]). Together, these activities support survival and reduce injury with ischemia.

mK_{ATP} Channels

K_{ATP} channels were first discovered in 1983 by A. Noma in a patch clamp study using cardiac muscle membrane preparations [63]. In 1997, Garlid and co-workers presented the evidence that mK_{ATP} channels have a cardioprotectve role in ischemia and reperfusion, and were a component in the mechanism for preconditioning [64]. A prototypical mK_{ATP} channel, as reviewed by Aguilar-Bryan and Bryan [65], is an octameric structure consisting of 4 sulfonylurea receptor (SUR1 or SUR2) subunits and four K^+ inward-rectifying (Kir6.1 or Kir6.2) subunits. Attached to the SUR subunits are two nucleotide binding domains (NBD; Figure 3). The mK_{ATP} channel is activated in low energy states by ADP, binding to NBDs, allowing the influx of K^+ into the mitochondrial inner matrix. Conversely, the mK_{ATP} channel is inhibited in high energy states when ATP closes the Kir channel. In the brain, it appears the predominant subtypes are SUR2 and Kir6.2, although the SUR1 and Kir6.1 subunits are present in smaller amounts [66]. The mK_{ATP} channel may trigger postconditioning via mechanisms dependent on matrix volume stabilization, respiratory inhibition, controlled production of ROS and the closure of the mPTP.

The physiological functions of mK_{ATP} channels have been debated. The activities of the mK_{ATP} channel and the K^+/H^+ exchanger are believed to maintain K^+ homeostasis within mitochondria by controlling mitochondrial volume and

moderating the outer-to-inner pH gradient needed to drive ATP synthesis. In the presence of hypoxia, whole cell pH decreases and ATP production declines. This causes a switch to anaerobic metabolism. A decrease in pH combined with an increase in the AMP/ADP ratio secondary to ATP metabolism causes the mK_{ATP} channel to open allowing the influx of K^+ into the inner matrix. This, in turn, activates the K^+(out)/H^+(in) exchanger decreasing the hydrogen gradient between the outer membrane and inner matrix [67]. By doing so, the proton motive force driving ATP production is attenuated and mitochondria energetics slow. During this time, the mPTP is closed as membrane stability and electrical potential are better maintained by the simultaneous activity of the mK_{ATP} channel and the K^+/H^+ exchanger. In addition, ROS generation is proportional to the availability of oxygen and activation of mK_{ATP} channel appears to *moderate* ROS production [58, 68]. However, this effect is only protective to a limited extent.

As anaerobic metabolism continues in response to an extended period of severe hypoxia, ATP hydrolysis exceeds ATP generation causing a dramatic rise in H^+ within the cell and mitochondrial inner matrix. At some point H^+ entry becomes lethal as it exceeds the outward pumping capacity of the mitochondrial electron transport chain already hindered by anaerobic metabolism. This results in a total loss of proton motive force driving ATP production. If prolonged, this loss results in osmotic matrix swelling, mitochondrial degradation, and release of apoptotic proteins such as cytochrome C.

Abrupt reperfusion following prolonged ischemia results in a substantial amount of ROS generation. More importantly in intact but vulnerable cells, ATP levels begin to rise, mK_{ATP} channels close and K^+ transport into the mitochondrial matrix declines. This indirectly decreases the activity of the K^+/H^+ exchanger (K^+ out / H^+ in). As H^+ ions are rapidly removed from the matrix during mitochondrial respiration, the inner matrix quickly alkalinizes, causing the mPTP to open [46]. Opening the mPTP rapidly elevates inner matrix osmotic pressure leading to matrix distension and if allowed to remain open mitochondrial rupture.

In total, mK_{ATP} channel closure associated with abrupt reperfusion could result in the significant elevation of ROS and increase the mitochondrial inner matrix osmotic pressure, causing the mitochondria to quickly swell or rupture, releasing apoptotic factors such as cytochrome C [39]. It is reasonable to suggest that maintaining the patency of the mK_{ATP} channel would be beneficial during reperfusion. Allowing the mK_{ATP} channel to remain open during reperfusion could: 1) moderate the generation of ROS, 2) reduce osmotic force within the matrix by promoting ion exchange, and 3) reduce the activity of the mPTP thereby providing a protective effect.

Activation of mK_{ATP} channels have been shown to be protective during reperfusion in cardiac and brain tissue [69-72]. Obal and colleagues [70] demonstrated the utility of inhaling volatile anesthetics as a postconditioning trigger through mK_{ATP} channel activation. In rats subjected to cardiac ischemia, postconditioning was invoked by administering 1 minimum alveolar concentration

(MAC) of sevoflurane for 2 minutes with the onset of reperfusion. This resulted in a significant decrease in cardiac infarct size. Penna and colleagues [69], isolated rat hearts and exposed them to an ischemic period followed by reperfusion. Their results suggested that postconditioning mechanisms are activated by a bradykinin or a DZ mechanism resulting in the upregulation of protein kinase G (PKG). This upregulation was dependent on early ROS generation triggered by mK_{ATP} channel activation. They emphasized that their results were different from mechanical manipulations by showing that pharmacological agents, such as bradykinin or DZ, administered during the reperfusion period could induce protection. ROS also regulate the activity of heat shock proteins (HSPs). Using an *in vitro* vascular smooth muscle preparation, Madamanchi and colleagues [73] discovered that the application of H_2O_2 significantly upregulated HSP70.

The Mitochondrial Permeability Transition Pore

The existence of the mPTP was confirmed in 1992 in rat liver mitoblast membranes [74]. The primary components of the mPTP (Figure 4) are the voltage-dependent anion channel in the outer membrane, the adenine nucleotide translocator, and the cyclophilin D protein within the matrix [75]. In general, it is thought that the opening of the mPTP occurs with a decrease in the inner matrix potential, decreased AMP and ADP levels, increased matrix Ca^{2+}, with alkalinization, or during oxidative stress [76]. mPTP opening blocks ATP formation and allows for the equilibration of small molecules [76, 77]. mPTP opening increases osmotic forces within the mitochondria inner matrix and leads

to degradation of the matrix membrane, causing the release of apoptotic factors, especially cytochrome C [78]. Also, as the mitochondrial membrane potential is perturbed, ATP synthase reverses its primary function and serves as an ATPase further depleting cellular ATP concentrations and increasing H^+ levels.

Feng and colleagues [79] determined that volatile anesthesia-induced postconditioning prevented the opening of the mPTP by inhibiting glycogen synthase kinase 3β (GSKβ). This activation was a result of PI3K-AKT signaling pathway activation with the resulting phosphorylation and inactivation of GSKβ, which protected against reperfusion damage. Argaud and colleagues [80] found that mechanical postconditioning decreased cellular Ca^{2+} and protected in vivo rabbit hearts suggesting that the mPTP could be inhibited by the PI3K-AKT-eNOS cascade. Bopassa and colleagues [81], using a rat heart preparation undergoing postconditioning, concluded that PI3K signaling regulates the closure of mPTP. In addition, Cohen, Yan, and Downey [82] further observed that postconditioning prevented mPTP opening as a result of inhaled CO_2-induced acidosis during the first minutes of reperfusion. They suggested that low cellular pH inhibits the opening of mPTP in heart tissue, but as the cellular pH normalizes, the inhibition of mPTP is lost. They hypothesized that by maintaining the cellular pH at a lower level while introducing oxygen during reperfusion, it was possible to keep the mPTP closed allowing the redox signaling necessary to trigger preconditioning-like protection. Cohen, Yan, and Downey suggest that moderate acidosis during reperfusion might be protective. This hypothesis was addressed through the use of sodium bicarbonate ($NaHCO_3$) during

postconditioning. In isolated rabbit hearts, acidic CO_2 perfusate at the time of reperfusion mimicked postconditioning while an alkaline $NaHCO_3$ perfusate blocked that effect. They hypothesized that an acidic environment inhibited mPTP opening while an alkaline environment favored mPTP opening. Fujita [83] also hypothesized that $NaHCO_3$ would blunt the protective properties of postconditioning. Using in vivo dog hearts that underwent ischemia, the administration of $NaHCO_3$ during four intermittent cycles of 1-min reperfusion with 1-min reocclusion of a coronary vessel completely abolished the postconditioning effects. Their results suggested that postconditioning leads to the opening of mK_{ATP} channels as a result of decreased pH, leading to the attenuation of cardiac infarct size. Wu and colleagues [71] directly examined the roles of mPTP and the mK_{ATP} channel in postconditioning. Using preconditioning and postconditioning in a rat model of cerebral stroke, Wu and colleagues activated the mK_{ATP} channel with DZ 20 min before middle cerebral artery occlusion followed by reperfusion, or inhibited the mPTP by infusion of cyclosporin A 15 min before reperfusion. It was discovered that both measures significantly increased functional performance scores and reduced infarction volumes. Importantly, both of these effects were abolished by blocking the adenine nucleotide port located on the mPTP. Their results strongly suggested that the mK_{ATP} channel and mPTP activity during reperfusion shared a common protective pathway.

The Reperfusion Injury Survival Kinase Pathway

The Reperfusion Injury Survival Kinase (RISK) pathway begins with the activation of PI3K and ERK to promote cell survival. RISK can be activated by insulin, urocortin, atorvastatin, adenosine, bradykinin, opioid agonists, volatile anesthetics, or DZ [84-91]. The RISK pathway promotes pro-survival signaling while inhibiting pathways associated with apoptosis. In 2004, Tsang and colleagues reported in isolated rat hearts, which had undergone mechanical postconditioning following an period of ischemia, that postconditioning is mediated by the PI3K-AKT-eNOS/p70s6K pathway [92]. They also suggested MEK 1/2-ERK 1/2 pathways were indirectly involved. Zhu and colleagues [93] followed by finding that cardioprotection from postconditioning in the remodeled rat myocardium is regulated through PI3K-AKT signaling. The role of ERK 1/2 was addressed by Darling and colleagues [94] and Krolikowski and colleagues [95]. Darling and colleagues utilized mechanical postconditioning in isolated rabbit hearts and found ERK1/2 but not PI3K activity provided cardiac protection. Krolikowski and colleagues exposed rabbits to isoflurane before and during early reperfusion and suggested a central role of ERK1/2, p70s6k, and eNOS in anesthetic-induced postconditioning.

Downstream in the RISK pathway, phosphorylation of AKT occurs with the subsequent phosphorylation of protein kinase C (PKC) and GSKβ. When PKC is phosphorylated it is stimulated while the phosphorylation of GSKβ inhibits its

activity. In a rabbit model, Philipp and colleagues [96] demonstrated through inhibitor studies that adenosine, PKC, and PI3K mediated the effects of mechanical postconditioning. In their investigation they concluded that protection was conferred through the activation of adenosine receptors by endogenous adenosine, a cellular metabolite. This in turn activated the PI3K component of the RISK pathway resulting in activation of PKC. In regards to GSKβ, Feng and colleagues [79], using isoflurane as a postconditioning trigger along with an AKT inhibitor, showed that when inhaled early in reperfusion, isoflurane phosphorylated AKT and GSKβ. Phosphorylated GSKβ was inhibited and could not promote the opening of mPTP. Feng and colleagues, using isoflurane as a postconditioning trigger, profiled the RISK pathway and determined that while the PI3K-AKT signal was strong, the ERK1/2-p38 MAPK was not altered. This suggests a primary role of PI3K-AKT in the RISK pathway and in mPTP closure. Recently, in human tissue, it has been found that the cytoprotective HSP25 and HSP70 are upregulated by the PI3K-AKT pathway [62].

Very recently in our laboratory, O'Sullivan and colleagues [72] demonstrated utility of DZ in a rat model of hemorrhage and cerebral hypoperfusion. In that investigation, DZ upregulated two stress proteins, HSP25 and HSP70. HSP25 and HSP70 can be regulated by signaling ROS or the RISK cascade and play key roles in providing cytoprotection [97-99]. O'Sullivan and colleagues demonstrated that DZ used as a preconditioning trigger and during the early reperfusion period increased the expression of HSP25 and or HSP70.

Heat Shock Proteins

The term "heat shock protein response" can be defined as the induction of heat shock or stress protein (HSP) synthesis that occurs in eukaryotic cells subsequent to heat treatment or exposure to other proteotoxic stress. This response appears to be a result of nonnative proteins accumulating in a stressed cell resulting in increased expression of HSPs [100]. The initial discovery of the heat shock response occurred in 1962. *Drosophilia* larvae, when heated, developed puffing patterns in certain chromosomal regions. This suggested a change in the synthetic activity of the chromosomal bands concerned [101]. Sixteen years later, the RNA for *Drosophilia* exposed to a thermal stimulus was coded using hybrid-arrested translation and indicated that proteins of 83, 72, 70, 68, 28, 26, 23 and 22 kilodaltons were upregulated [102]. Over the following decades, the investigation of the heat shock response has confirmed that a family of highly conserved HSPs is upregulated following a variety of sublethal stressors. These proteins are subcategorized by their molecular weight and are either inherently present or can be induced following sublethal stress. In particular, HSP25 and HSP70 have been thoroughly investigated with the consensus being they are protective when upregulated following stress [103-105].

Heat Shock Protein 25

HSP 25 is the rodent equivalent of the primate HSP27 and often the terms are used interchangeably. HSP27 confers protection at different levels as it can interact with several proteins implicated in cell death based upon its phosphorylation and oligomerization condition and not upon ATP. The main mechanisms for how HSP27 confers cytoprotection appear to be: molecular chaperoning, interference with cell death pathways, signaling of antiapoptotic pathways, stabilization of the cytoskeleton, and antioxidant activities. Serving as a chaperone, HSP27 can bind folded intermediate non-native proteins, inhibiting their aggregation, and in the presence of HSP70 these HSP27 bound proteins can be reactivated [106]. Within the cytosol, HSP27 can sequester cytochrome C interfering with the formation of the apoptotic protease activating factor-1 (APAF-1)-cytochrome c multimeric apoptosome and the activation of procaspase 9 [107-109]. HSP27 also directly precipitates with procaspase-3 decreasing the activity of activated caspase-3 [109]. HSP27 serves as a signaling messenger by causing the activation of serine/threonine kinase Akt thereby inhibiting Bcl-2 and caspase-9 [110]. Phosphorylated HSP27 can stabilize F-actin and increase the number of cells retaining microfilament organization thus stabilizing membrane structure [111] . Additionally, HSP27 is able to increase glutathione levels thereby reducing levels of ROS [112].

Heat Shock Protein 70

. Over the last three decades, HSP70 has become the most thoroughly investigated protein of the HSP family of proteins. Like HSP25, HSP70 can

inhibit cell death at various sites within the cell. However, unlike HSP25, HSP70 function is “ATP-dependent”. HSP70 is typically found *in vivo* bound by ATP and HSP70 function is typically based upon the hydrolysis of the attached ATP molecule. HSP70 serves as a chaperone protein, inhibits stress signaling, prevents mitochondrial membrane permeabilization, and inhibits apoptotic pathways. HSP70 may chaperone kinases by binding to an unfolded carboxyl terminus, preventing aggregation, and allowing re-autophosphorylation of the kinase enzyme; thus stabilizing the enzyme and restoring function [113]. HSP70 also binds the death receptors, DR4 and DR5, inhibiting Apo-2L/TRAIL-induced cell death [114] and HSP70 blocks Bax translocation into the mitochondrial outer membrane. The latter effect prevents the permeabilization of the mitochondrial membrane and subsequent release of apoptosis inducing factor (AIF) and cytochrome C [115]. HSP70 binds AIF within the cytosol inhibiting its nuclear translocation and limiting nuclear condensation [116]. Similar to HSP25, HSP70 prevents cell death by binding to Apaf-1 and interfering in the formation of the apoptosome complex and subsequent recruitment of procaspase-9 [99]. Lastly, HSP70 suppresses apoptotic signaling by binding precursor forms of caspase-3 and caspase-7 preventing their cleavage and activation [117].

Cleaved Caspase–3 (CC3)

Both HSP25 and HSP70 inhibit the cleavage of Caspase-3 [109, 117]. Cleaved Caspase-3 (CC3) is a primary executioner of apoptosis as it is responsible for the total or partial proteolytic cleavage of numerous key cellular

survival proteins [118]. One being the abundant nuclear enzyme poly-polymerase, which functions in DNA repair and protein modification during oxidative stress [119]. Thus, induction of HSP25 and HSP70 may alleviate cerebral ischemic injury and resuscitation injury that results from the mitochondrial release of cytochrome C with subsequent cleavage of caspase-3.

Contrasting Evidence in Postconditioning

There is scarce evidence that disputes the numerous findings that postconditioning is beneficial. In 2005, for example, Lucchinetti and colleagues [120] suggested that postconditioning suppressed the expression of many HSP genes. The authors noted, though, that caution should be used when interpreting the data. Alterations in mRNA levels do not always correlate with protein expression and they proposed that future studies should evaluate genomic and proteomic responses to postconditioning triggers. Schwartz and Lagranha [121] using mechanical postconditioning in a pig model found that although postconditioning resulted in the phosphorylation of ERK1/2 and AKT, it did not have a protective effect for pig hearts subjected to an ischemic period. The authors used a postconditioning design that occluded a coronary vessel for 3 cycles of 30 seconds. However, the pig is a much larger species than used in previous models and some human evidence suggests that longer cycles of vessel occlusion in larger species may be required for protection. In support of this statement, Staat and colleagues [122] found in patients undergoing angioplasty there was a 36% reduction in serum CK levels suggesting a

protective effect had occurred. In their human study, postconditioning was elicited using four episodes of 1 minute inflation followed by 1 minute deflation. Recently, three relevant abstracts appeared in *The Journal of Molecular and Cellular Cardiology* [123-125]. Kupai and colleagues [123] suggested that the cardioprotective effects of postconditioning are lost in a hyperlipidemic state where peroxynitrite is elevated. Kocsis and colleagues [124] concluded that chronic statin treatment abolishes postconditioning effects. Finally, Boengler and colleagues [125] showed in aged mice, postconditioning is absent. What the findings of Kupai, Kocsis, and Boengler have in common is a model of chronic disease. In the absence of prior modifications to improve the health of the organism it is not remarkable that postconditioning is ineffective.

Clinical Utility

Two general postconditioning maneuvers can be implemented for the treatment of hemorrhagic or occlusive injuries (i.e., massive trauma, stroke, TBI): 1) episodic clamping and unclamping of the vasculature, and 2) alteration of the reperfusion environment. The clinical realm in which on-site postconditioning is most applicable is in the cardiac setting. Routine procedures such as myocardial revascularization or angioplasty are performed where the surgeon has the ability to control flow through the on-site vasculature. Thus in that context, organ protection could be promoted through the use of a mechanical postconditioning protocol prior to allowing complete uninterrupted reperfusion. Originally, postconditioning involved strict protocols of alternating episodes of clamping and

unclamping of the vasculature prior to reperfusion at the site of injury. Since then an additional technique termed remote-postconditioning [126, 127] has been described where the application of a mechanical clamping/unclamping protocol applied to a distant organ or extremity can reduce tissue injury. Remote-postconditioning could have wider utility, especially in the operative environment in which most surgeries result in the hypoperfusion of the area of interest. This maneuver could be employed during the majority of surgical procedures through the use of pneumatic tourniquets applied to the upper or lower extremities and programmed for intermittent inflation/deflation. This would result in the remote activation of postconditioning triggers without impinging on the surgical field.

Even more promising and far reaching is the ability to exogenously alter the reperfusion fluid prior to restoring flow. This technique can be safely and easily implemented in nearly all clinical situations and would be more practical than the application of physical methods of induction. Exogenous alterations can occur by the addition of a pharmacological agent to the reperfusion fluid or lowering the pH of the fluid and/or environment. For example, it has newly been established in animal models that agents such as opioids, adenosine, volatile anesthetic agents, and DZ can be used in fluid therapy as a postconditioning trigger and decrease tissue injury. But clinically, this technique has not received much attention. It appears that postconditioning could be clinically used on a far-reaching degree, and this leaves a great deal of opportunity for further research. The results thus far suggest that DZ may be beneficial in treating casualties who suffer a traumatic injury that involves severe hemorrhage when given in

conjunction with resuscitative efforts. DZ could be carried by medics on the battlefield or civilian first responders and administered via intramuscular (IM) or intravenous (IV) injection as part of IV resuscitation. This intervention could decrease cerebral cellular injury and improve outcomes.

Research Goals

Hemorrhagic shock has long been a feature of civilian and military trauma and the leading cause of death from trauma-related injury. Trauma victims, many times, suffer a concurrent brain injury as a result of blunt force or ischemia. The loss of circulating blood volume from hemorrhage decreases the perfusion pressure necessary to provide the metabolic substrates required for aerobic metabolism within the brain. Ischemic injury occurs and numerous biochemical processes result in apoptosis and necrosis, organ failure, and ultimately severe impairment or death. For decades, researchers have investigated resuscitative strategies to improve the outcomes of trauma victims. Initial actions are deemed vital for the survival of injured persons—blood loss must be controlled and intravascular volume re-established. Too often, however, the resuscitative measures employed throughout the echelons of care are unsuccessful and, as reviewed earlier, resuscitation itself is not completely benign. The long-term goal of this research is to investigate resuscitative interventions that may decrease neurological injury and dysfunction resulting from hemorrhagic shock and improve morbidity and mortality. The specific aims of this investigation were to 1) assess the capacity of DZ IV resuscitation to elicit the expression of

cytoprotective HSP25 and HSP70 within the hippocampus and cortex, when DZ is given before, at the time of, or after resuscitation of laboratory rats that have sustained hemorrhagic shock and cerebral ischemia (Chapter 3); and 2) survey brain regions for injury following hemorrhagic shock and cerebral ischemia, and determine whether DZ treatment before, at the time of, or after resuscitation, attenuates injury (Chapter 4).

Experimental Approaches

Taken together, postconditioning by the activation of mK_{ATP} channels is an interesting prospect for inducing neuroprotection from ischemia-reperfusion injury. As outlined below, the goal of this research is to assess the impact of DZ, a prototypical mK_{ATP} channel opener upon brain injury induced by cerebral hypoperfusion from hemorrhagic shock and unilateral carotid artery occlusion. For this investigation, a model of hemorrhagic shock with concomitant cerebral ischemic injury was used. Anesthetized laboratory rats underwent a 40% blood loss through the right carotid artery followed by permanent ligation of the right carotid artery. DZ was administered at three time points following the cessation of hemorrhage, either 40 minutes after, 60 minutes after, or 80 minutes after hemorrhage; time points that were 20 minutes before, at the time of, or 20 minutes after the initiation of resuscitation, respectively. These time points were based upon average evacuation times cited by military and civilian trauma services following mass casualty events. During combat, evacuation times to higher level care average 35 to 40 minutes while in the civilian setting urgent

evacuation times have averaged 44 +/- 26 minutes [128, 129]. Experimental resuscitation began 60 minutes following hemorrhage. Western blotting was conducted to determine the expression of the protective markers HSP25 and HSP70 within the hippocampus and cortex. Immunohistochemistry was utilized to assess the location and characteristic cell types for HSP25 and HSP70 (Chapter 3) as well as assess cell death and damage with the injury markers cleaved caspase-3 (CC3) and glial fibrillary acidic protein (GFAP) (Chapter 4). **It is hypothesized that postconditioning with DZ will increase HSP25 and HSP70 levels while decreasing CC3 and GFAP levels in the hypoperfused cerebral cortex and hippocampus ipsilateral to the carotid occlusion.**

Figure 1. The sphere of injurious effects secondary to reperfusion injury

Reperfusion causes a vigorous increase in oxygen tensions promoting the rapid and persistent generation of reactive oxygen species (ROS). Dormant ATP-dependent processes are activated augmenting the loss of ATP. Cytosolic and mitochondrial calcium levels dramatically rise. The mitochondrial Permeability Transition Pore (mPTP) opens allowing an influx of usually impermeable protein and ions into the mitochondrial matrix. A broad endothelial dysfunction & general cell lysis occurs and there is a generation of microvascular flow defects (no-reflow phenomenon).

Figure 1. The sphere of injurious effects secondary to reperfusion injury

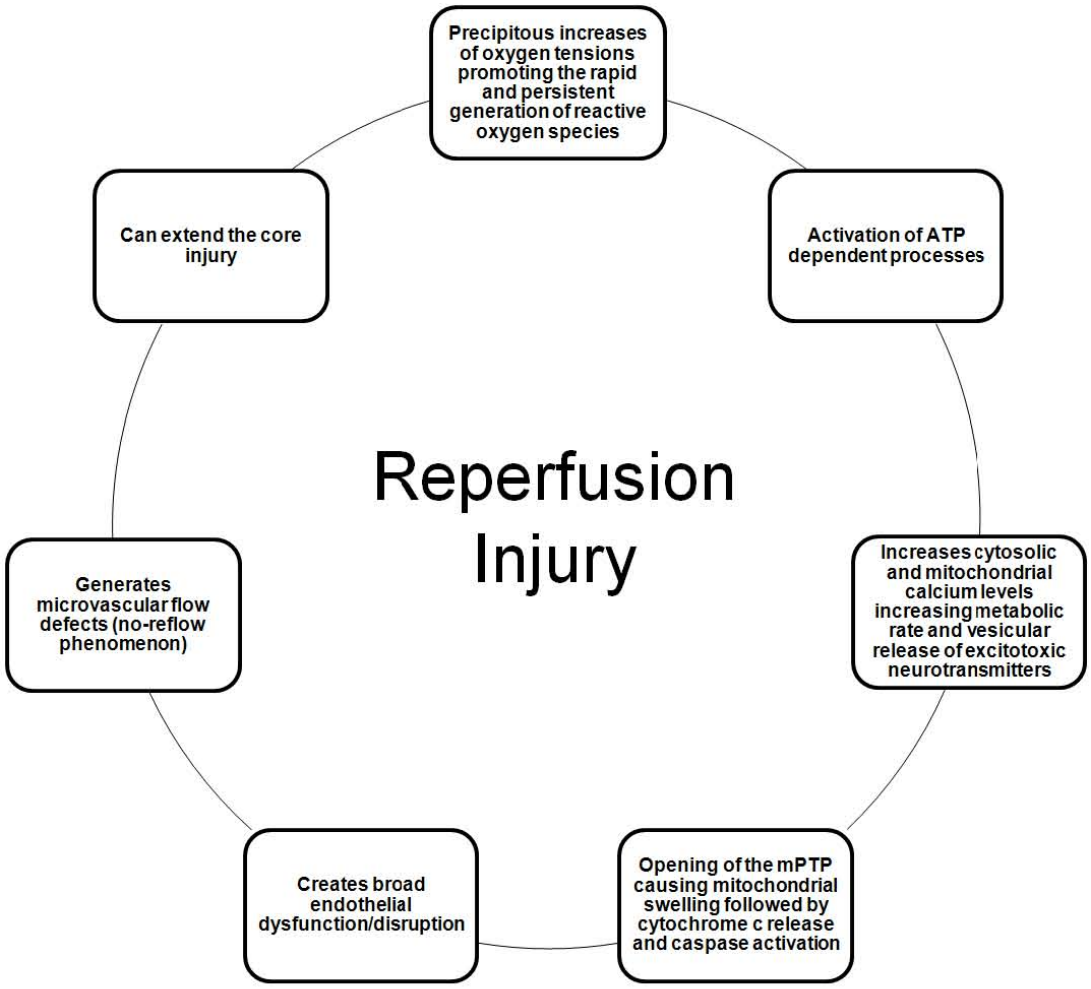


Figure 2. Schematic of how diazoxide (DZ) may invoke postconditioning.

Postconditioning with diazoxide (DZ), acting upon mK_{ATP} channels, increases mitochondrial matrix K^+ and matrix volume as obligate water follows K^+. As matrix pH rises, proton and electron motive forces slow. Superoxide ion is produced by Complex I. Superoxide dismutase then generates reactive oxygen species (ROS; represented here as H_2O_2), which promotes the activation of prosurvival kinases, closes the mitochondrial permeability transition pore (mPTP), and enhances the opening of mK_{ATP} channels. Together, these activities support survival and reduce injury. (Arrows indicate activation while bar indicates inhibition. OM – outer mitochondrial membrane; IM – inner mitochondrial membrane)

Figure 2. Schematic of how diazoxide (DZ) may invoke postconditioning.

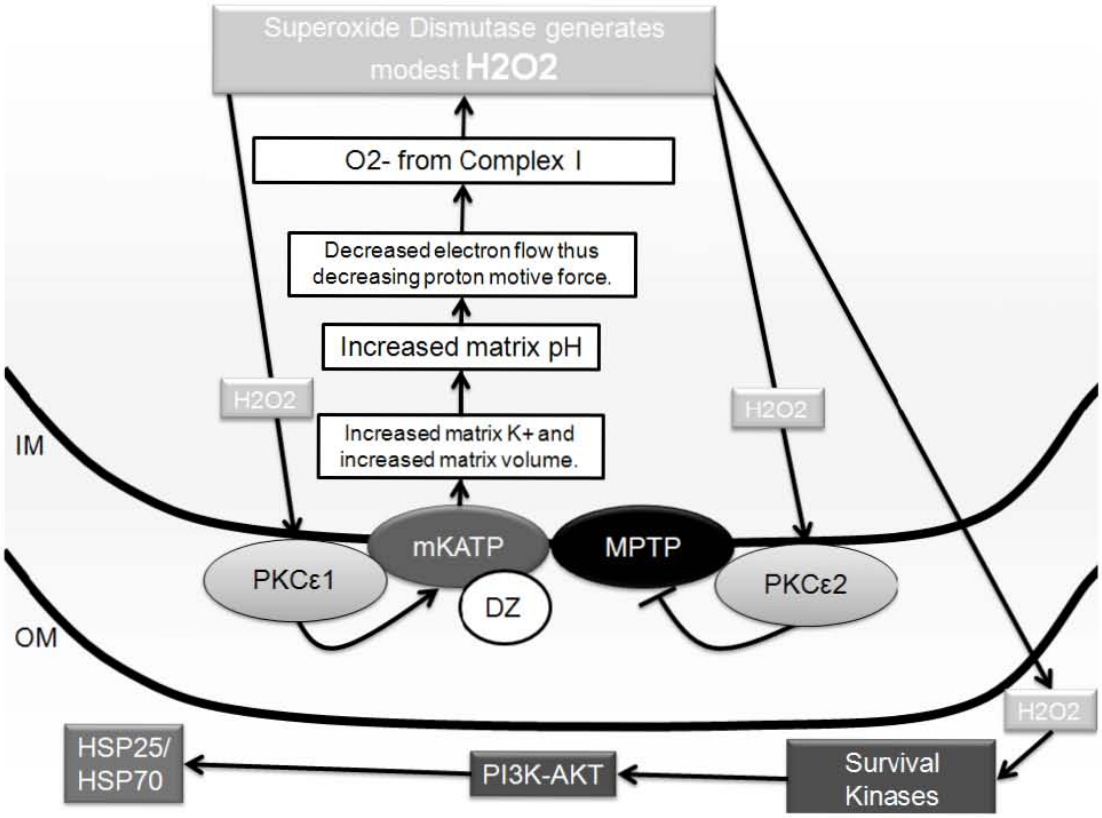


Figure 3. The mitochondrial ATP-sensitive potassium (mK_{ATP}) channel.

A prototypical mK_{ATP} channel is an octameric structure consisting of four sulfonylurea receptor subunits (SUR1 or SUR2) and four K^+ inward-rectifying subunits (Kir6.1 or Kir6.2). Attached to the SUR subunits are two nucleotide binding domains (NBD). mK_{ATP} channels are activated in low energy states by ADP, binding to NBDs, allowing the influx of K^+ into the mitochondrial inner matrix and, conversely, mK_{ATP} channels are inhibited in high energy states when ATP closes the Kir channel.

Figure 3. The mitochondrial ATP-sensitive potassium (mK_{ATP}) channel.

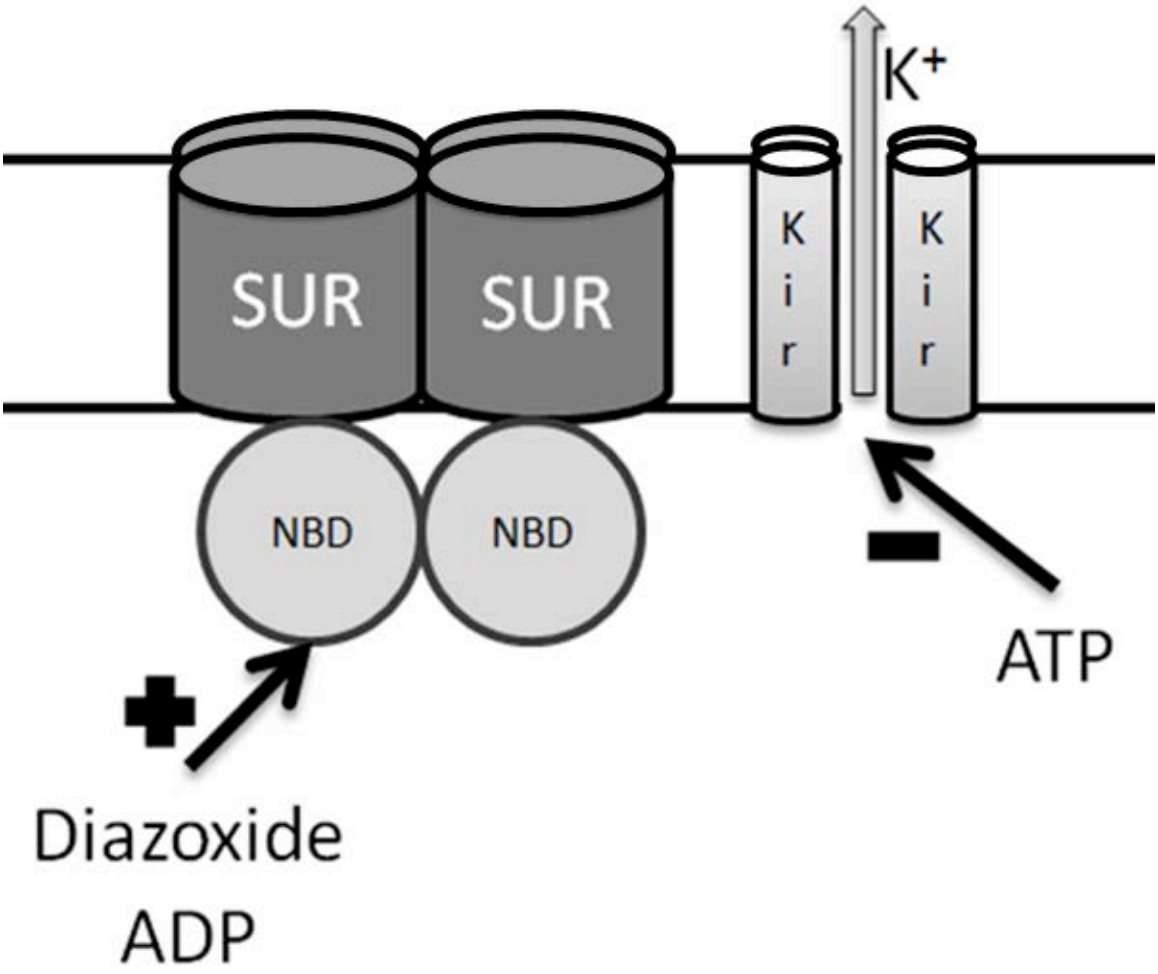
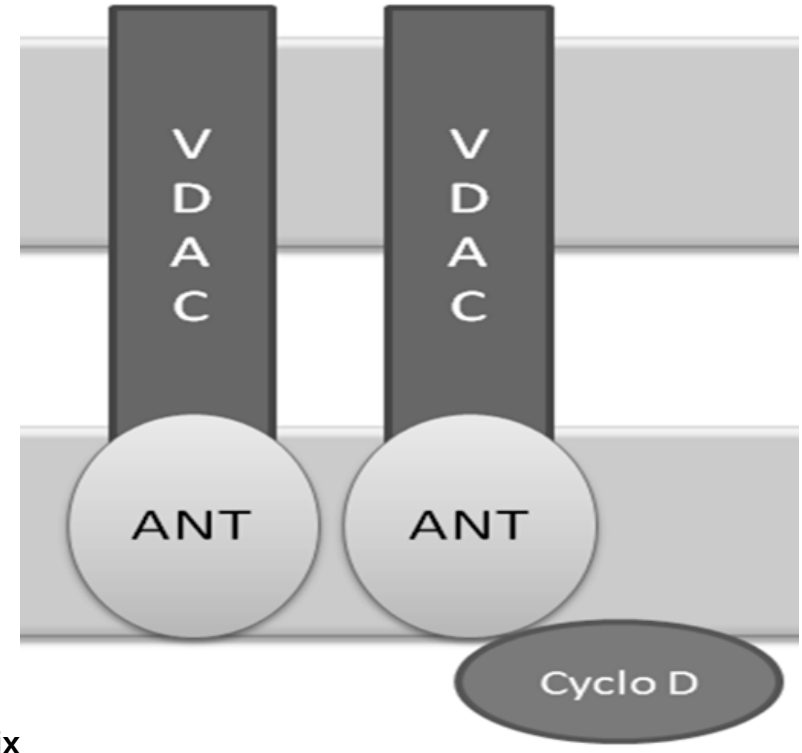


Figure 4. The mitochondrial Permeability Transition Pore (mPTP).

The primary components of the mPTP are the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator (ANT), and the cyclophilin D protein within the mitochondrial inner matrix. In general, it is thought that opening of the mPTP occurs with a decrease in the inner matrix potential, decreased AMP and ADP levels, increased matrix Ca^{2+}, with alkalinization, and during oxidative stress.

Figure 4. The mitochondrial Permeability Transition Pore (mPTP).



Inner Matrix

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CHAPTER 2

OVERVIEW OF EXPERIMENTAL DESIGN AND METHODS

Experiment 1: The Effect of Diazoxide on the Expression of HSP25 and HSP70 within the Hippocampus and Cerebral Cortex when given as part of IV Resuscitation subsequent to Hemorrhagic Shock with concomitant Cerebral Ischemic Injury.

Experiment 2: The Effect of Diazoxide on attenuating Cellular Injury within the Hippocampus and Cerebral Cortex when given as part of IV Resuscitation subsequent to Hemorrhagic Shock with concomitant Cerebral Ischemic Injury.

Groups

Male, Sprague-Dawley rats (200-475 gm body weight, Charles River Laboratories) were quartered for 48-72 hours and had *ad libitum* access to rat chow and water. Animals were randomly assigned to one of nine groups (Table 1): Group 1 animals served as a control in which, with the exception of euthanasia for tissue retrieval and processing, animals did not undergo any surgical or experimental manipulations. Group 2, the Sham group, underwent anesthesia, an incision was made from the area of the mandibular salivary gland to the sternum, and the right carotid artery (RCA) was exposed through blunt dissection. Group 3, the Unilateral Carotid Artery (UCAO) group, the animals underwent anesthesia and the RCA was exposed and cannulated for arterial blood pressure monitoring (ABP) and arterial blood gas analysis (ABG). Group

4, the UCAO Vehicle (UCAO VEH) group, the animals underwent anesthesia, and the RCA was cannulated for ABP and ABG. The caudal vein was cannulated for peripheral intravenous access to allow the infusion of 1 ml of 0.9% normal saline (NS) vehicle. In Group 5, the UCAO DZ group, animals underwent anesthesia, the RCA was cannulated for ABP and ABG, and the caudal vein was cannulated for IV access for the infusion of 3.2 mg/kg of DZ in 1 ml 0.9% NS. Group 6 was the Hemorrhagic Shock Cerebral Hypoperfusion (HSCH) group. These animals received anesthesia, the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for resuscitation (0.9% NS given at 3 times the blood loss). Group 7 consisted of the animals who received DZ (3.2 mg/kg IV) 40 minutes following the exsanguination of 40% BV (DZ40 Group) but 20 minutes prior to the resuscitation (0.9% NS given at 3 times the blood loss). These animals received anesthesia and the RCA was cannulated for ABP, ABG, and exsanguinations. The caudal vein was cannulated for intravenous access and resuscitation. Group 8 (DZ60 Group) was identical to the DZ40 Group, but received DZ (3.2 mg/kg IV) simultaneously with resuscitation (0.9% NS given at 3 times the blood loss). Group 9 (DZ80 Group) was identical to the DZ40 and DZ60 Groups, but received DZ (3.2 mg/kg IV) 80 minutes following the loss of 40% BV and 20 minutes after the beginning of resuscitation (0.9% NS given at 3 times the blood loss). The dose of DZ was selected based upon previous work by our laboratory [1, 2].

Model

Table 1 (pg. 64) summarizes the groups for the procedure. An induction chamber (5% Isoflurane) was used to induce unconsciousness and induction tested by the loss of response to tail clamp. Animals were then weighed and placed on the sterile surgical field and maintained on spontaneous ventilation with 1% Isoflurane via a nose cone apparatus connected to a passive scavenging system. A rectal thermometer connected to a Hewlett Packard Merlin Multi-Parameter Monitor was inserted and temperature was maintained between 36.5–37.5^{o}C by a radiant heat lamp. A small incision was made midway along a line from the right mandibular salivary gland to the sternum and a point of entry was located in the triangle formed by the sternohyoideus, sternomastoideus, and masseter muscles. These muscles were retracted by blunt dissection. The RCA was gently cleared from the vagus nerve and the proximal end was permanently ligated with sterile 3-0 silk thread. A sterile 24 gauge angiocatheter was then inserted in a caudal fashion into the RCA and then connected to a three-way stopcock that was connected to a primed arterial blood pressure (ABP) extension. The ABP transducer was then connected to the Hewlett Packard Merlin Multi-Parameter Monitor. The 24 gauge angiocatheter was then secured to the vessel with 3-0 silk thread. At that point a tourniquet was placed at the most proximal portion of the tail and a scissor incision was made over the anterolateral portion of the tail. The caudal vein was identified and cannulated with a sterile 24 gauge angiocatheter for peripheral IV access. The tourniquet was released, blood flow was verified and the catheter was

flushed with 0.5 ml NS to ensure patency. The IV was then saline-locked and secured to the tail using 3-0 silk thread. Three 18 gauge needles were then placed through small folds of skin on the right upper extremity, the left upper extremity, and the left lower extremity and connected to the Hewlett Packard Merlin Multi-Parameter Monitor electrocardiogram (ECG) leads via ECG clips. This allowed the monitoring of heart rate and respirations. The placement of lines and monitors took approximately 30 minutes.

After placement of the lines and monitors, the time was set to zero. A baseline recording of vital signs was recorded and vital signs were documented every 5 minutes. After the first five minutes, animals in Groups 6-9 received a controlled hemorrhage of 40% blood volume. Blood volume was calculated in milliliters (ml) with the following equation: BV = (0.06 X body weight in grams) + 0.77 [3]. At time 5 minutes, 0.3 ml of blood was removed for ABG and the remaining 40% of blood was extracted over 10 minutes. Arterial blood gases were analyzed using either a Stat Profile 2 Blood Gas and Electrolyte Analyzer or a Stat Profile Ultra Blood Gas and Electrolyte Analyzer (Nova Biochemical, Waltham, MA). After ten minutes, a second ABG was drawn for 0.3 ml of blood. To ensure accuracy, all blood sponges were weighed as each gram of blood equals 1 ml of blood [4]. Following the exsanguination, the animals remained in a shocked, non-resuscitated state for 60 minutes. DZ40 animals received DZ (3.2 mg/kg in 1 ml 0.9% NS) through the caudal vein 40 minutes following hemorrhage, which was 55 minutes into the procedure. At seventy-five minutes (60 minutes following hemorrhage), a third ABG of 0.3 ml blood was taken and

resuscitation was started. In the first ten minutes of the resuscitation phase, animals received 0.9% NS based upon the following formula: first ten minutes resuscitation volume (FTMRV) = body weight in kilograms X 28.57 ml/kg. This mimics the early administration of 2000 ml of lactated Ringer's solution (based upon an average 70kg male human) as recommended by the ATLS guidelines [5]. The remaining resuscitation volume was then pro-rated over the remaining 35 minutes of the procedure. If the animals were in the DZ60 group, they received 3.2 mg/kg of DZ at Time 75 minutes (60 minutes subsequent to exsanguinations) mixed in with the first 10 minute resuscitation volume. At time 95 minutes (80 minutes following hemorrhage), the DZ80 group received 3.2 mg/kg DZ IV in 1 ml 0.9% NS given over ten minutes concurrently with the remaining pro-rated resuscitation fluid. At time 120 minutes, a final ABG using 0.3 ml blood was taken. The RCA catheter was removed and the tie securing the catheter was tightened and the vessel was permanently ligated. The incision was approximated using either staples or 3.0 silk thread, the venous catheter was removed and incision approximated with two staples. The ECG leads were removed and the animals were placed back into their cage in a lateral recovery position. The procedure ranged between 2½ to 3 hours in length and 2-3 animals were administered this model per day. After recovery, the rodents were returned to their home cages for 24 hours.

Western Blotting

In Experiment 1, western blots were performed to measure changes in HSP25 and HSP70 (relative to ß-actin levels). Four separate areas of each animal (right and left cerebral cortices, right and left hippocamppi) were homogenized in lysis buffer (10X phosphate buffered saline (PBS)-50ml, 10% SDS-5ml, NP-40 5ml, sodium deoxycholic acid-2.5 grams and 460ml ddH2O) with protease and phosphatase inhibitors (Roche Laboratories) with a sonicator. Lysates were centrifuged at a setting of 12,000 for 20 minutes at 4^{o}C. Total protein concentration was determined for each sample by measuring the ultraviolet absorbance at a wavelength of 595 nm compared to a standard curve using known protein concentration samples. Fifty micrograms of total protein from each sample and loading buffer were boiled for 5 minutes and then loaded on Nu-Page gels (Invitrogen) and separated by electrophoresis. Proteins were transferred to nitrocellulose membranes using the IBLOT transfer system (Invitrogen). Membranes were blocked with 5% dry milk in lysis buffer, and then incubated with the appropriate concentration of primary antibody (Stressgen: HSP25 SPA801 or HSP70 SPA-810; 1:5,000 and 1:2,000 concentrations, respectively) overnight at 4^{o}C on a shaker table. The membranes were washed in washing buffer, and then incubated with the species appropriate HRP-conjugated secondary antibody at 1:10,000 for HSP25 and 1:5,000 for HSP70 for 1 hour at room temperature. The membranes were washed and incubated in chemiluminescent detection reagents (Millipore) for 5 minutes and images captured using Fuji LAS 1000 software. The relative protein levels were

determined from the optical densities of the corresponding protein bands after subtraction of background values obtained from the same lane. In addition, all samples were normalized to the amount of β-actin protein by reprobing each blot for β-actin. Western blot membranes were prepared in one of four configurations for each tissue region analyzed. The first configuration consisted of Group 1 (Control), Group 2 and Group 3. The second consisted of Group 1, Group 4 and Group 5. The third configuration was Group 1, Group 6 and Group 7. Finally, the fourth consisted of Group 1, Group 8 and Group 9. Comparisons between membranes were performed by standardizing the samples in each configuration to the mean band intensity of the Group 1 samples on that membrane.

Immunohistochemistry

For Experiments 1 and 2, immunohistochemistry methods were employed. Twenty-four hours after surgery, rats were anesthetized and transcardially perfused with 100mM PBS followed by 4% paraformaldehyde in PBS. Whole brains were removed and post fixed in 4% paraformaldehyde in PBS at 4^{o}C overnight then transferred into 30% sucrose. Frozen 20µm thick cerebral coronal sections were cut via a cryostat and placed in cryoprotectant and stored at –20^{o}C. Sections in the locality of coordinates interaural 5.40 mm and bregma -3.60 mm [6] were floated onto slides submersed in gelatin and chromium potassium sulfate. Slides were dried on a slide warmer at 50^{o}C for ten minutes and then stored at –20^{o}C. After slides were removed from storage, they were placed in 10% formalin for 30 minutes and underwent an antigen retrieval

processing. Slides were placed in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 100^{o}C for 10 minutes and washed in tap water for 20 minutes. The slides were then placed in blocking solution for one hour at room temperature followed by washing in PBS/0.2% Triton X-100. Primary antibodies (Experiment 1: HSP25 SPA801 1:100 and HSP70 SPA-810 1:50, (Stressgen); Experiment 2: CC3 #9661 1:50, (Cell Signaling) and GFAP #1665-1 1:100, (Epitomics)) in 1:100 goat serum/PBS/0.3% Triton were placed on slides for 2 hours at 37^{o}C. Following washing in PBS/0.2% Triton, a species appropriate fluorescently conjugated secondary antibody (1:200 for HSP25 and GFAP; 1:100 for HSP70 and CC3) in 1:100 goat serum/PBS/0.3% Triton was placed on slides for 1 hour at 37^{o}C. Slides were washed and then mounted with a hard mount containing DAPI stain. Images of cerebral cortex and hippocampus were obtained at 200x or 50x using an Axioplan inverted microscope and Axiovision software (Zeiss).

Fluorojade C

Stored frozen slides were removed and placed in 4% paraformaldehyde or 10% formalin for 20 minutes. Slides were washed in 10mM PBS and dried on a slide warmer at 50^{o}C for 10 minutes. Slides were then immersed in 1% NaOH/80% ethanol followed in sequence by 2 minutes in 70% ethanol, 2 minutes in dH_2O and 5 minutes in 0.06% $KMnO_4$, followed by rinsing in dH_2O for 2 minutes. Under low light conditions, slides were placed in 0.0001% Fluorojade C in 0.1% acetic acid for 10 minutes. After that, slides were rinsed in dH_2O and

dried on a slide warmer at 50^{o}C for 10 minutes. Slides were then mounted with DPX and images obtained at 50x using an Axioplan inverted microscope and Axiovision software (Zeiss).

Statistical Analysis

The SigmaStat 3.1 (SPSS) statistical analysis program was used to assess group differences using a one-way analysis of variance and *post hoc* testing using the Holms-Sidak method. If preliminary tests indicated the data sets did not meet the assumptions for normality distribution or homogeneity of variance, the Kruskal-Wallis one-way analysis of variance was employed, followed by a post hoc Dunn's test. This method was used to determine differences between each experimental group for HSP 25 and HSP70 from western blotting in Experiment 1. For Experiment 2, one-way analyses of variance (ANOVA), followed by post hoc Holm-Sidak tests was applied to determine differences in CC3 and GFAP for the immunohistochemistry of 200x images in the cerebral cortex and hippocampus. If normality or variance tests failed, the Kruskal-Wallis (KW) ANOVA was performed.

A Fisher exact test was performed on HSP 25 and HSP70 immunohistochemistry results in Experiment 1. Observations were made under 50x magnification for the presence or absence of staining and categorized as "1" for present or "0" for absent. For assessing changes in hematological and physiological parameters (i.e. mean blood pressure, heart rate, arterial blood gas

results), ANOVAs were employed and involved a two-factor Treatment x Trials design with the second factor “Trials” considered a repeated measure.

Table 1. Experimental Groups

Groups	Treatment
Control	The animals did not endure any anesthetic, surgical or experimental manipulations.
Sham	Anesthesia performed and an incision was made from the area of the mandibular salivary gland to the sternum. The RCA was exposed through blunt dissection.
UCAO	Anesthesia performed, the RCA was exposed and cannulated for ABP and ABG.
UCAO VEH	Anesthesia performed, and the RCA was cannulated for ABP and ABG. The caudal vein was cannulated for peripheral IV access to allow the infusion of 1 ml of 0.9% NS vehicle.
UCAO DZ	Anesthesia performed, the RCA was cannulated for ABP and ABG. The caudal vein was cannulated for peripheral IV access for the infusion of 3.2 mg/kg of DZ in 1 ml 0.9% NS.
HSCH	Anesthesia performed, the RCA cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation of 0.9% NS given at 3 times the blood loss.
DZ40	Anesthesia performed and the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV 40 minutes following the hemorrhage of 40% BV but 20 minutes prior to the resuscitation.
DZ60	Anesthesia performed and the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes., The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV at the onset of resuscitation.
DZ80	Anesthesia performed and the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV 20 minutes after the onset of resuscitation.

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CHAPTER 3

POST-HEMORRHAGIC PRE-RESUSCITATION CONDITIONING USING DIAZOXIDE INCREASES HSP25 AND HSP70 IN THE CEREBRAL CORTEX AND HIPPOCAMPUS

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Abstract

Hemorrhagic shock resulting from injury is the most common cause of traumatic death in both civilian and military settings. In addition, the majority of victims have a coinciding head injury. Resuscitative measures that are useful subsequent to injury are few. As a result, our team investigated the hypothesis that diazoxide (DZ), a mitochondrial K_{ATP} channel opener, could be used during resuscitation to induce a postconditioning effect in a laboratory animal model of hemorrhagic shock with concomitant cerebral ischemic injury. Male Sprague-Dawley rats sustained a unilateral right common carotid artery occlusion followed by a 40% total blood loss volume. Resuscitation commenced one hour later, where DZ was administered at one of three time points: 20 minutes before the onset of resuscitation, at the time that resuscitation began, or 20 minutes after the initiation of resuscitation. Western blotting showed that when DZ was administered intravenously 20 minutes prior to resuscitation, heat shock protein (HSP) 25 and HSP70 exhibited marked upregulation within the ipsilateral cerebral cortex and hippocampus at 24 hours following injury. . Immunocytochemistry for HSP25 and HSP7 was used to determine cellular expression after shock and the DZ treatment 20 minutes before resuscitation. Ipsilateral to the carotid artery occlusion, intense imunostaining was observed in the parietooccipital cerebral cortex, hippocampus, thalamus, hypothalamus, amygdala, and temporal cortex. Dual-immunolabeling showed the expression of HSP25 and HSP70 was focal, where HSP25-positive cell profiles, astrocyte-like in appearance, formed a border surrounding HSP70-positive cell profiles that

appeared neuron-like. In the cortex thalamus, and CA2 and CA3 regions of the hippocampus, neuron-like cell profiles were also occasionally observed that immunostained for both antigens. Taken together, a model of cereral ischemic injury from hemorrhagic shock demonstrated that DZ treatment prior to shock resuscitation induces a robust expression level of HSP25 and HSP in focal regions of the neuraxis, ipsilateral to common carotid artery occlusion. Further work will determine whether HSP expression from DZ resuscitation is related to improved morphological and functional outcome.

Key Words: Diazoxide, Hemorrhagic Shock, Postconditioning, Brain, Resuscitation, Heat Shock Proteins

INTRODUCTION

Injury and metabolic stresses are a constant threat to cell function and survival. To reduce the lethal effects of injury and environmental stresses such as hypoxia, extreme temperatures, and starvation, organisms have evolved mechanisms that permit cells and organs to withstand permanent damage and increase the organism's overall viability. Over the years, several fields of investigation have recognized these cellular and organismic processes and begun to elucidate their signal transduction and metabolic pathways. One field that has made significant advances is cardiac physiology, which has demonstrated evidence for intrinsic pathways that lead to cytoprotection from oxygen and metabolic dysregulation. Investigators have firmly established the phenomenon of "preconditioning" in which techniques are employed to increase cellular defenses before the occurrence of ischemic injury. Exposure to a range of "mild" stressors was found to increase cellular defenses before the occurrence of ischemic injury [1]. However, the clinical relevancy of preconditioning is limited as it is difficult to predict and integrate interventions prior to injury. "Postconditioning" is similar to preconditioning; however, the critical factor that separates the two maneuvers is the temporal relationship for the deployment of the protective intervention. Postconditioning refers to the activation of cytoprotective mechanisms either through mechanical or pharmacological means *subsequent* to hemorrhage or an occlusive, ischemic injury but prior to reperfusion, and can thus be employed after an ischemic injury.

The roots of postconditioning stem from the work of Okamoto and colleagues in 1986 [2]. Dogs, in which a coronary vessel was clamped, were reperfused either with the sudden release of a coronary occlusion, or by low-pressure (40 to 50 mm Hg) coronary reperfusion with blood for 20 minutes before completely releasing the occlusion. This maneuver focused on the initial stage of reperfusion and established that with timely, low-pressure, gentle reperfusion post-ischemic damage could be limited. The term, “postconditioning,” was described in the literature in 2003 [3]. Using dogs as their animal model, Zhao and colleagues, determined that when a 60-minute occlusion of the left anterior descending (LAD) artery was followed by 3 cycles of 30-second vessel release with 30-second re-occlusion prior to full release of the LAD, there was a significant 44% reduction in cardiac infarct area. This was validated by Kin and colleagues in 2004 [4] who determined the first minute of reperfusion in the rat was crucial for postconditioning. Following a 30-minute occlusion of the left coronary artery (LCA), 3 cycles of 10 second LCA release, with 10 second LCA re-occlusion preceding full LCA occlusion release, reduced cardiac infarct size by 23%.

The significance of postconditioning lies in its temporal relationship to ischemic injury as it can be implemented following an insult that customarily results in cell and tissue ischemia. This is exceptionally relevant for the treatment of unexpected traumatic injuries. Traumatic injury resulting in hemorrhage and consequential hypoxia is the most significant cause of death in both the military and civilian settings [5-7]. In addition, 64% of hemorrhagic

fatalities have a coinciding brain injury [8] either directly from the primary traumatic event or as a result of secondary injury.

Postconditioning appears to be triggered through the activation of mitochondrial potassium-ATP (mK_{ATP}) channels as the effect can be abolished through the use of mK_{ATP} antagonists such as 5-hydroxydecanoic acid [9]. Postconditioning effects may also be linked to the activation of the Reperfusion Ischemia Survival Kinase Pathway and expression of protective heat shock proteins [10-19]. Postconditioning can be pharmacologically induced following an ischemic event but prior to reperfusion [20]. Mizumura and colleagues subjected dogs to a 60-minute occlusion of the LAD followed by 3 hours of reperfusion. Using bimakalin, an mK_{ATP}-sensitive channel opener, it was discovered that if bimakalim was given after an ischemic period, but prior to reperfusion, injury could be significantly reduced. Recently, Xing and colleagues found that mechanical postconditioning could limit cerebral infarct volumes and increase the expression of protective heat shock proteins if employed following a period of ischemia but prior to reperfusion [21]. Finally, our team has demonstrated that pharmacologic postconditioning using diazoxide shows potential as a neuroprotective postconditioning trigger for hemorrhagic shock [19]. The use of mK_{ATP} agonists such as diazoxide as part of resuscitative efforts following a traumatic, hemorrhagic injury may be beneficial in decreasing cerebral injury and improving mortality. Since postconditioning may be protective when implemented *subsequent* to a traumatic injury resulting in hemorrhage, our team investigated the use of diazoxide in a rat model of hemorrhagic shock with

concomitant ischemic brain injury. DZ was administered at one of three time points following hemorrhagic shock, either 40 minutes after, 60 minutes after, or 80 minutes after hemorrhage. These first two time points were based upon average evacuation times cited by military and civilian trauma services following mass casualty events. During combat, evacuation times to higher level care average 35 to 40 minutes while in within the civilian setting urgent evacuation times have averaged 44 $\pm$ 26 minutes [22, 23]. The 80 minute timepoint was selected to assess the utility of DZ after aggressive resuscitation measures had been initiated.

The measurement of HSPs was used as a marker of cytoprotection. Based upon the evidence of their functions, HSP25 is the rodent equivalent of the primate HSP27 and often the terms are used interchangeably. HSP25 and HSP70 can provide protection at different levels as they can interact with several proteins implicated in cell death. HSP25 confers cytoprotection via: molecular chaperoning, interference with cell death pathways, signaling of antiapoptotic pathways, stabilization of the cytoskeleton, and antioxidant activities. [24-30]. HSP70 serves as a chaperone protein, inhibits stress signaling, prevents mitochondrial membrane permeabilization, and inhibits apoptotic pathways. [31-36]

MATERIALS AND METHODS

One hundred-thirty four Male Sprague-Dawley rats (200 – 445 grams) from Charles River Laboratories (Wilmington, MA) were quartered for 48-72

hours with a 12-hour light/dark cycle, constant temperature (24°C), and given unlimited access to water and rat chow. All procedures were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, Bethesda, Maryland.

Experimental Groups

A postconditioning paradigm was employed for two experiments utilizing the intravenous administration of DZ given at three different time points following hemorrhagic shock with concomitant cerebral ischemic injury (Figure 1). Experiment 1 employed western blotting to evaluate the impact of DZ upon the expression of the cytoprotective proteins, HSP25 and HSP70, in four regional brain samples: the right and left cerebral cortices and right and left hippocamppi. Experiment 2 was performed to observe the presence, localization, and cell-type characterization of HSP25 and HSP70 expression within the above noted regions of each animal.

Animals were randomly assigned to one of nine groups (Table 1). Group 1 animals served as a negative control. These animals did not undergo any surgical or experimental manipulations and were immediately euthanized for tissue retrieval and processing. Group 2 was a surgical sham control where animals underwent anesthesia and an incision was made from the area of the mandibular salivary gland to the sternum. The right carotid artery (RCA) was exposed and the vagus nerve cleared from the RCA through blunt dissection. Group 3, the Unilateral Carotid Artery Occlusion (UCAO) group, received

anesthesia and the RCA was bluntly dissected and exposed. The RCA was cannulated for arterial blood pressure (ABP) monitoring. Group 4, the UCAO Vehicle (UCAO VEH) group, received anesthesia and the RCA was bluntly dissected and exposed. The RCA was cannulated for ABP monitoring. The caudal vein was cannulated for peripheral intravenous access to allow the infusion of 1 ml of 0.9% normal saline (NS) vehicle 75 minutes into the procedure. In Group 5, the UCAO DZ group, received anesthesia and the RCA was bluntly dissected and exposed. The RCA was cannulated for ABP monitoring. The caudal vein was cannulated for peripheral intravenous access to allow the infusion of 3.2 mg/kg of DZ in 1 ml 0.9% NS 75 minutes into the procedure. Group 6 was the Hemorrhagic Shock Cerebral Hypoperfusion (HSCH) group. These animals received anesthesia, the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for the resuscitation of 0.9% NS given at three times the blood loss beginning 75 minutes into the procedure (60 minutes following hemorrhage). Group 7, consisted of animals that received anesthesia, the RCA was cannulated for ABP monitoring and 40% blood loss, and the caudal vein was cannulated for intravenous access and the resuscitation. These animals received DZ, 3.2 mg/kg IV, 40 minutes following the loss of 40% BV (DZ40 Group) but 20 minutes prior to resuscitation with 0.9% NS given at three times the blood loss. Group 8 (DZ60 Group) was identical to the DZ40 Group, but received DZ 3.2 mg/kg IV 60 minutes after hemorrhage and simultaneously with the onset of resuscitation. Group 9 (DZ80 Group) was identical to the DZ40 and DZ60 Groups, but received

DZ 3.2 mg/kg IV 80 minutes following the loss of 40% BV (20 minutes after the beginning of resuscitation). In all groups, following 120 minutes of anesthesia, the RCA was permanently ligated (Groups 3-9), wounds were approximated, the animals recovered, and then placed in their home cages. Twenty-four hours later, animals were euthanized and tissue processed according to the experimental protocol.

Drug Preparations

Anesthesia was induced, via a nose cone apparatus, with the halogenated methyl-ethyl-ether Isoflurane (Abbott Labs, Chicago) at 5% concentration in room air until loss of tail clamp response. Anesthesia was maintained with room air and 1% Isoflurane (0.89 minimum alveolar concentration, [37]) at all times except during hemorrhage in which a concentration of 0.6% Isoflurane (0.54 minimum alveolar concentration) was used. Prior to incision, 1 ml of 1% Lidocaine (Phoenix, St. Louis) was infiltrated into the area covering the mandibular salivary gland to the sternum. DZ (Sigma, St. Louis) was prepared by dissolving 10mg of DZ in 1ml of 0.5 μM NaOH and then diluted to 10 ml of NS for a final concentration of 1mg/ml. DZ was given at one of three time points in Experiments 1 and 2. If given 40 minutes following hemorrhagic shock, 3.2 mg/kg of the 1 mg/ml solution was given over 10 minutes. If given at the onset of reperfusion, 3.2 mg/kg of the 1 mg/ml solution was added in the resuscitation fluid and given over ten minutes. If given 20 minutes following the onset of resuscitation, DZ 3.2 mg/kg of the 1 mg/ml solution was injected through the

peripheral IV and given over 10 minutes. For euthanasia, intraperitoneal injections of 80 mg/kg Ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 10 mg/kg Xylazine (Phoenix, St. Louis) were used.

Surgical Protocol

Following isoflurane induction, the rats in Groups 2-9 were weighed and the area of the sternum extending to mandibular salivary was shaved and cleaned with 50% ethanol. The rat was then placed on a sterile surgical field. A rectal thermometer (Hewlett Packard Merlin Multi-Parameter Monitor) was inserted and temperature maintained by adjusting the height of a radiant heat lamp to keep rectal temperature between 36.5–37.5^{o}C. After infiltration with 1 ml of 1% Lidocaine, a neck incision was made and the triangle formed by the sternohyoideus, sternomastoideus, and masseter muscles located and muscles retracted. Through blunt dissection, the vagus nerve was cleared intact from the RCA. The proximal portion of the RCA was permanently ligated with sterile 3-0 silk thread. A sterile 24 gauge angiocatheter was introduced into the RCA and connected to a three-way stopcock-ABP extension. After connecting the ABP transducer to the monitor (Hewlett Packard Merlin Multi-Parameter Monitor), the angiocatheter was secured to the RCA with a 3-0 silk thread tie.

Following RCA cannulation, a tourniquet was placed at the base of the tail. A cut-down was performed to expose a caudal vein. A sterile 24 gauge angiocatheter was introduced into the vein for peripheral intravenous access. Following tourniquet release, a 3 ml syringe filled with 1 ml NS was connected.

Blood was withdrawn to verify placement and the IV was saline-locked with 0.5ml NS. Electrocardiographic recordings and respiratory rates were obtained by using three 18 gauge needles placed through small pinches of skin on the right upper extremity, the left upper extremity, and the left lower extremity, which were then clipped to ECG leads (Hewlett Packard Merlin Multi-Parameter Monitor). This process was typically performed in 30–45 minutes.

Following a 5 minute baseline recording of vital signs, animals in Groups 6-9 received a controlled hemorrhage of 40% blood volume. Blood volume was calculated with the following equation: BV in ml = 0.06 X (body weight in grams) + 0.77 [38]. Prior to the initiation of hemorrhage an ABG was taken. ABG analysis was conducted using either a Stat Profile 2 Blood Gas and Electrolyte Analyzer or a Stat Profile Ultra Blood Gas and Electrolyte Analyzer (Nova Biochemical, Waltham, MA). At the conclusion of hemorrhage, a second ABG was performed. Following hemorrhage, rats remained in a shocked, non-resuscitated state for 60 minutes. At this time point a third ABG was performed and the first phase of resuscitation initiated. In the first ten minutes of resuscitation, rats received 0.9% NS based upon the following formula: first ten minutes resuscitation volume (FTMRV) = body weight in kilograms X 28.57ml/kg. FTMRV mimics the aggressive administration of 2 liters of lactated Ringer's solution based upon an average 70kg male human as recommended by the ATLS guidelines [39]. Following FTMRV, the second phase of resuscitation consisted of pro-rating the remainder of resuscitation volume over the last 35 minutes of procedural time so that the total resuscitation volume equaled 3 times

the amount of blood lost. At the conclusion of the procedure, a 4th ABG was taken, the RCA catheter removed, and the RCA was ligated with a second 3.0 silk tie. Incisions were closed using either staples or 3.0 silk thread. The tail vein catheter was removed and the cut-down site approximated. ECG leads were retrieved and the rats recovered in the surgical suite. Following recovery, rats were transported to their home cages. The procedure ranged between 2½ to 3 hours in length and 2-3 animals were administered this protocol per day.

Western Blotting

In Experiment 1, Western blots were performed to evaluate changes in HSP25 and HSP70 within the right and left cerebral cortices and hippocamppi. Tissue was homogenized in lysis buffer (10X PBS-50 ml, 10% SDS-5 ml, NP-40 5 ml, sodium deoxycholic acid-2.5 g and 460 ml ddH2O) with protease and phosphatase inhibitors (Roche Laboratories) and further disrupted via sonication. Lysates were centrifuged at a setting of 12,000 for 20 minutes at 4^{o}C. Total protein concentration per sample was determined by measuring ultraviolet absorbance at a wavelength of 595 nm compared to a standard curve of known protein concentration. Fifty micrograms of protein per sample was prepared in loading buffer, boiled for 5 minutes, and loaded on Nu-Page gels (Invitrogen). Following electrophoretic separation, gel transfer onto nitrocellulose membranes occurred using the IBLOT transfer system (Invitrogen). Membranes with 5% dry milk in lysis buffer were blocked and incubated overnight with the primary antibody (Stressgen: HSP25 SPA801 or HSP70 SPA-810; 1:5,000 and 1:2,000

concentrations, respectively) with agitation at 4^{o}C. The next day, membranes were incubated with the species appropriate HRP-conjugated secondary antibody concentration (1:10,000 for HSP25 and 1:5,000 for HSP70) for 1 hour at room temperature. After using chemiluminescent detection reagents (Millipore), images were captured using Fuji LAS 1000 software.

. **Immunohistochemistry**

For Experiment 2, immunohistochemistry methods were employed to observe the presence, localization, and cell-type characterization of HSP 25 and HSP 70 in the cerebral cortex and hippocampus of both hemispheres. Twenty-four hours following surgery, rats were anesthetized and transcardially perfused with 100mM phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Whole brains were retrieved and placed in 4% paraformaldehyde in PBS overnight at 4^{o}C. The following day the brains were transferred into 30% sucrose in PBS for 1-3 days and then stored at -80^{o}C. Coronal sections (20µm) were retrieved using a cryostat, placed in cryoprotectant and transferred to storage at –20^{o}C. Sections in the area of coordinates interaural 5.76 mm, bregma -3.24 mm [40] were free-floated onto gelatin-chromium potassium sulfate submersed slides. Slides were dried and then stored at –20^{o}C. Following slide fixation with 10% formalin for 30 minutes, antigen retrieval processing took place. Slides were heated in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 100^{o}C for 10 minutes and washed in water for 20 minutes. Following blocking for one hour at room temperature, slides were incubated with

primary antibodies (HSP25 SPA801 1:100 and HSP70 SPA-810 1:50, Stressgen) for 2 hours at 37^{o}C. Following washing, slides were incubated for 1 hour at 37^{o}C with species appropriate fluorescently conjugated secondary antibodies. Following mounting, images of the cerebral cortex and hippocampus were obtained using 5x and 40x magnification with an Axioplan inverted microscope coupled with an Axiovision camera and software (Zeiss).

Statistical Analysis

The relative protein levels of HSP25 and HSP70 were determined from the optical densities of the corresponding protein bands after subtraction of background values obtained from the same lane. All samples were normalized to the amount of β-actin protein by re-probing each blot for β-actin. The mean density of the Control group on each membrane was calculated and divided into each sample yielding ratio difference values. The data from each sample was then transformed to natural logarithm values if the assumption of homogeneity of variance was violated. The SigmaStat 3.1 (SPSS) statistical analysis program was used to assess group differences using a one-way analysis of variance and *post hoc* testing using the Holms-Sidak method. If preliminary tests indicated the data sets did not meet the assumptions for normality distribution or homogeneity of variance, the Kruskal-Wallis one-way analysis of variance was employed, followed by a *post hoc* Dunn's test. All histograms summarize the data in terms of the geometric mean $\pm$ SEM of each group.

For immunohistochemistry analysis, observations were made under 5x magnification for the presence or absence of HSP staining and categorized as “1” for present or “0” for absent. Fisher exact tests were performed to compare the number of animals of each group for relative staining for HSP expressions.

RESULTS

HSP25 and HSP70 expression in the cerebral cortex and hippocampus

When given 40 minutes subsequent to hemorrhage but 20 minutes prior to the onset of resuscitation (DZ40), DZ yielded a significant elevation of HSP25 in the right cerebral cortex and right hippocampus compared to that observed in samples from animals in the HSCH group ($p < 0.001$ and 0.016, respectively). All other group comparisons in the right cerebral cortex to the HSCH group found no change in HSP25 levels (Figures 2 and 4). When assessing the left cerebral cortex and left hippocampus western blot data, there were no differences between the HSCH group and levels seen in the other treatment groups.

A similar change was observed for HSP70. The administration of DZ 40 minutes after shock and cerebral hypoperfusion (DZ40 Group) significantly elevated HSP70 levels when compared to the HSCH results ($p < 0.001$ for the cerebral cortex and hippocampus; Figures 3 and 4). In the left hemisphere, two unexpected results occurred. Within the left cerebral cortex, the UCAO VEH group that received normal saline vehicle but no hemorrhage, had significantly elevated HSP70 expression compared to HSCH ($p = 0.006$; Figure 3). However

it appears to be a modest elevation. In addition, in the left hippocampus, HSP70 was significantly elevated in the group receiving DZ 80 minutes following shock and 20 minutes after the onset of resuscitation when compared to HSCH ($p < 0.001$; Figure 3), but this appears to be a modest elevation.

Immunohistochemistry Analysis of HSP25 and HSP70 in the Cerebral Cortex and Hippocampus.

Observations using immunostaining were employed to further characterize HSP changes after experimental treatment. In the right cerebral cortex, both HSP25 and HSP70 were robustly expressed in the DZ40 group compared to all other groups (Tables 2 and 3). The result was the same in the right hippocampus; HSP25 and HSP70 were highly expressed in the DZ40 group and significantly different when compared to all other groups (Tables 2 and 3). There were no significant differences in HSP25 or HSP70 staining in the left cerebral cortex or left hippocampus between groups (not shown).

A unique pattern of HSP expression in the right hemisphere was observed in the DZ40 Group. Within the cerebral cortex, HSP25-stained cell profiles were characteristic of astrocytes and bordered HSP70-stained cell profiles, which appeared to be neurons. However, colocalization of HSP25 and HSP70 was occasionally observed in what appeared to be neurons within the cerebral cortex (Figure 6).

In the hippocampus, HSP25 could be found in what appeared to be astrocytes and neurons. HSP25 had intense neuronal-like staining in the CA2 and CA3 regions of the hippocampus. HSP70 in the hippocampus appeared in neuronal-like cells and could be found in the CA1, CA2 and CA3 regions, with especially robust HSP70 staining in the neuronal-like cells of the CA2 and CA3 region. Dual immunofluorescence verified intense colocalization of HSP25 and HSP70 in the CA2 and CA3 pyramidal cell region (Figure 7).

Within the thalamus, HSP25 could be observed in the lateroposterior and laterodorsal thalamic nuclei in both astrocyte-like and neuronal-like cells. HSP70 was also observed in these nuclei but with a greater majority of staining in the laterodorsal thalamic nucleus in neuronal-like cells (Figure 8). Again, dual immunolabeling showed many neuronal-like cells with HSP25 and HSP70 colocalization. In the ventral diencephalon, HSP25 and HSP70 immunostaining was observed in the hypothalamus in the regions of the perifornical nucleus and the lateral hypothalamic region within the medial forebrain bundle (Figure 10). The pattern characteristics were similar to those found in the cerebral cortex where HSP25-positive cell profiles formed a border surrounding HSP70-positive cells. In this region HSP25 appeared in astrocyte-like cells while HSP70 could be found in neuronal-like cells.

Another region where HSP25 cells formed a border surrounding HSP70 cells was in the temporal area. In a region that began dorsally within the external capsule, extended down to the rostral amydalopiriform cortex, and to the

posterodorsal med ial amygdaloid nucleus, HSP25 profiles also bordered HSP70 staining regions. Again, HSP25 appeared in this region to be mainly in astrocytic-like cells, while HSP70 was observed primarily in neuronal-like cells (Figure 9).

DISCUSSION

Model

To our knowledge, this is the first report of a model of hemorrhagic shock that utilizes the right carotid artery for hemorrhage and the tail vein for intravenous resuscitation. This model produced a consistent unilateral injury ipsilateral to the occlusion (Figure 11). This is not surprising as Mitchell and Himwich [41] found on average a 73% drop in blood pressure to the right lingual artery and an average 46% reduction in the left lingual artery of the rat following the occlusion of the RCA. This finding demonstrates the impact of a single carotid occlusion on collateral flow. This animal model permits robust survival of rats 24 hours post hemorrhage, as 72 of 76 rats who lost 40% of their blood volume and were resuscitated survived. Of the four animals that did not survive 24 hours post surgery, one was in the HSCH group, one in the DZ80 group, and two in the DZ60 group. Previous investigation in our lab [42] had found significant differences in survival of animals undergoing shock versus those that did not. However, that model involved surgical trauma to the right lower extremity for arterial and venous access in conjunction with RCA occlusion. The improvement in survival seen here emphasizes the detrimental consequences of

coexisting traumatic injury when combined with brain insult. The consequences are vividly evident when analyzing the multi-center clinical databases in the United Kingdom. These databases show a doubling of the risk of death when moderate brain injury is combined with hemorrhagic shock and/or tissue injury [8]. Thus, the avoidance of surgical extremity trauma in this model generated 94.7% survival in animals that received a 40% hemorrhage from the right carotid artery followed by its permanent occlusion and resuscitation via the tail vein. Using this model, the main result of this investigation revealed the robust upregulation of HSP25 and HSP70 in the right cerebral cortex and right hippocampus in the DZ40 group.

Diazoxide and the Heat Shock Response

Diazoxide is a nondiuretic benzothiadiazine with antihypertensive properties, which has been used in the treatment of blood pressure since the early 1960s [43]. Diazoxide is a mK_{ATP} and plasma membrane K_{ATP} channel opener and has shown a 2,000-fold specificity for mK_{ATP} [44]. Diazoxide also inhibits cellular respiration through the inhibition of cytochrome II and attenuates reactive oxygen species release and those actions are dependent upon the energy state of the cell [45, 46]. Previous research has suggested that DZ acting on mK_{ATP} can: increase mitochondrial matrix K^+ and volume, increase the elevation of matrix pH, decrease proton and electron motive forces, generate beneficial signaling of ROS, which catalyzes the activation of prosurvival kinases, close mPTP, and cyclically enhance the opening of mK_{ATP} channels [18, 44, 47-

50]. The actions of DZ, then, may postcondition the ischemic injury while preconditioning reperfusion injury.

First described in 1962 [51], the “Heat Shock Response” occurs in eukaryotic cells following exposure to a proteotoxic stress and features the induction of heat shock or stress protein (HSP) synthesis [52]. HSP25 and HSP70 are two of the most investigated HSPs and are protective when upregulated following stress [53-55]. HSP25 confers protection by serving as a molecular chaperone, by its interference with cell death pathways, promotion of signaling of antiapoptotic pathways, stabilization of the cytoskeleton, and by its antioxidant activities [24-29]. HSP70 provides protection as it is a chaperone protein, inhibits stress signaling, prevents mitochondrial membrane permeabilization, and inhibits apoptotic pathways [31-36] . The synthesis of these HSPs in the DZ40 group solely, suggests that the pre-resuscitation administration of DZ provides the most benefit for cellular defenses.

The reason for why DZ can provoke such a marked response may be as simple as delivery of DZ to the site of injury. These results suggest that DZ must be available at the site of ischemic injury prior to the onset of reperfusion. When DZ is given in the DZ40 group, MAP has recovered on average to 50mmHg, which may be adequate to restore a perfusion pressure of at least 13-14 mmHg to the right cerebral circulation in a rat with a unilateral carotid artery occlusion [41]. This pressure may be adequate to deliver DZ to the sites of injury.

Another consideration is that reperfusion is not benign. Restoration of flow and/or resuscitation can increase oxygen tensions promoting generation of ROS, increase cytosolic and mitochondrial calcium levels, open the mitochondrial Permeability Transition Pore (mPTP), and generate microvascular flow defects (no-reflow phenomenon) [56]. In this paradigm, there is a primary ischemic injury followed by a secondary reperfusion insult. Without having DZ at the site of action prior to resuscitation, cells may not have the inherent capability to survive the secondary insult. This could account for the lack of protein response in the HSCH, DZ60, and DZ80 groups.

Finally, how can DZ increase HSP25 and HSP70 when given after hemorrhagic shock but prior to resuscitation? DZ has been demonstrated to activate the PI3K-AKT signaling pathway [50] and, if presented to a vulnerable but viable cell prior to a secondary insult, this pathway may be stimulated. The PI3K-AKT signaling pathway has been directly linked to the expression of HSP25 and HSP70. In human esophageal endothelial cells, Rafiee and colleagues [18] found that both HSP25 and HSP70 were upregulated by the PI3K-AKT signaling pathway in response to acidic stress. Additionally, a p38 MAPK inhibitor, which inhibits one arm of the PI3K cascade, significantly reduced both HSP25 and HSP70 in response to stress. It was also determined that when an AKT inhibitor was used, HSP70 was significantly downregulated in response to stress. Further *in vivo* assessments of PI3K-AKT pathway status just before and after shock, with and without DZ treatment, may further characterize early responses to cerebral hypoperfusion.

Patterns of HSP25 and HSP70 Expression

In the majority of observations, HSP25 appeared to be present mostly in astrocytic-like cells and HSP70 was present in neuronal-like cell profiles. These findings are in accordance with several previous studies following ischemic paradigms [57-63]. In addition, HSP25 consistently appeared to surround HSP70-positive cell profiles. It has been suggested that HSP70 neuronal staining is indicative of vulnerable cells, bordering the infarcted zone of injury, that will survive the ischemic insult [57], and HSP25 immunostaining has been previously described as delineating the "outer border" of a cerebral ischemic injury [61]. In addition to the border-like pattern of HSP25-positive cells surrounding HSP70-positive cells, there were instances of remarkable, intense colocalization of HSP25 and HSP70 in the CA2, CA2/3, and CA3 region. One study found intense HSP25 staining but that occurred in the CA1, neocortical, and thalamic regions following six minutes of ischemia but HSP70 was not addressed [60]. One possibility could be that certain cell phenotypes co-express these HSPs or that robust colocalization activity arises from a transfer of HSP25 produced in glial to neuronal cells with receptor binding of HSP25 and subsequent neuronal internalization [64, 65].

Within nervous system tissue, Latchman has found that HSP27 has a more protective effect than HSP70. His group overexpressed HSP27 or HSP70 in mice and found that, following middle cerebral artery occlusion, infarct volumes were smaller in the HSP27 mice. One thing is clear; both proteins have been

shown to be protective and were highly expressed in this investigation when DZ was given after an ischemic insult but prior to resuscitation.

Hemorrhagic shock and DZ treatment before resuscitation elicited HSP25 and HSP70 expression in other brain regions, including the thalamus, hypothalamus, amygdala, and piriform cortex. Interestingly, these areas are involved in many behaviors such as fear, aggression, mood, attention, sympathetic control and motivation and may play a role in neuropsychiatric disorders

Summary

In summary, when DZ, an mK_{ATP} channel opener, is administered intravenously following hemorrhagic shock but prior to intravenous resuscitation, the expression of the cytoprotective proteins, HSP25 and HSP70, are markedly increased. This is highly relevant as this investigation suggests that an intervention following injury may offer neuroprotection from both a primary ischemic injury and a secondary reperfusion injury. Further immunohistochemistry and behavioral work is warranted. This study did not address the effect of this paradigm on cell death in the brain nor did it evaluate cerebral blood flow dynamics. Evaluations 3-7 days post injury may also be necessary as cell death may not be evident within the first 24 hours. Additionally, functional outcomes are warranted as it appears that this paradigm affects several areas within subregions of the cerebral cortex, hippocampus, thalamus, hypothalamus, and amygdala.

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Figure 1. Hemorrhagic shock with concomitant cerebral injury protocol.

Animals were anesthetized and monitors/lines placed. Baseline readings of vital signs (heart rate, respirations, temperature, mean arterial pressure) were documented before induction of 40% volume controlled blood loss. Following hemorrhage, the animal was left in a shocked state for 60 minutes. After 60 minutes, the 1st phase of resuscitation occurs; the animals received 28.57 ml/kg of 0.9% NS to approximate a 2L infusion of fluid over the first ten minutes (First Ten Minutes Resuscitation Volume; FTMRV). 0.9% NS infusion continues (the 2^{nd} phase of resuscitation) over the next 35 minutes so that total replacement volume was three times the amount of blood loss during hemorrhage. Diazoxide (3.2 mg/kg IV) treatment was administered to certain groups at one of three timepoints (indicated by filled circles), either 40 minutes following hemorrhage (DZ40), at the time of the start of resuscitation (DZ60), or 20 minutes after resuscitation starts (DZ80). Body temperature was maintained via a rectal temperature probe and a radiant heat lamp. Vital signs were documented every 5 minutes. Arterial blood gases were drawn at 4 time points (indicated by asterisks): at the onset of hemorrhage, at the end of hemorrhage, at the beginning of resuscitation, and at the end of the surgical procedure.

Figure 1. Hemorrhagic shock with concomitant cerebral injury protocol.

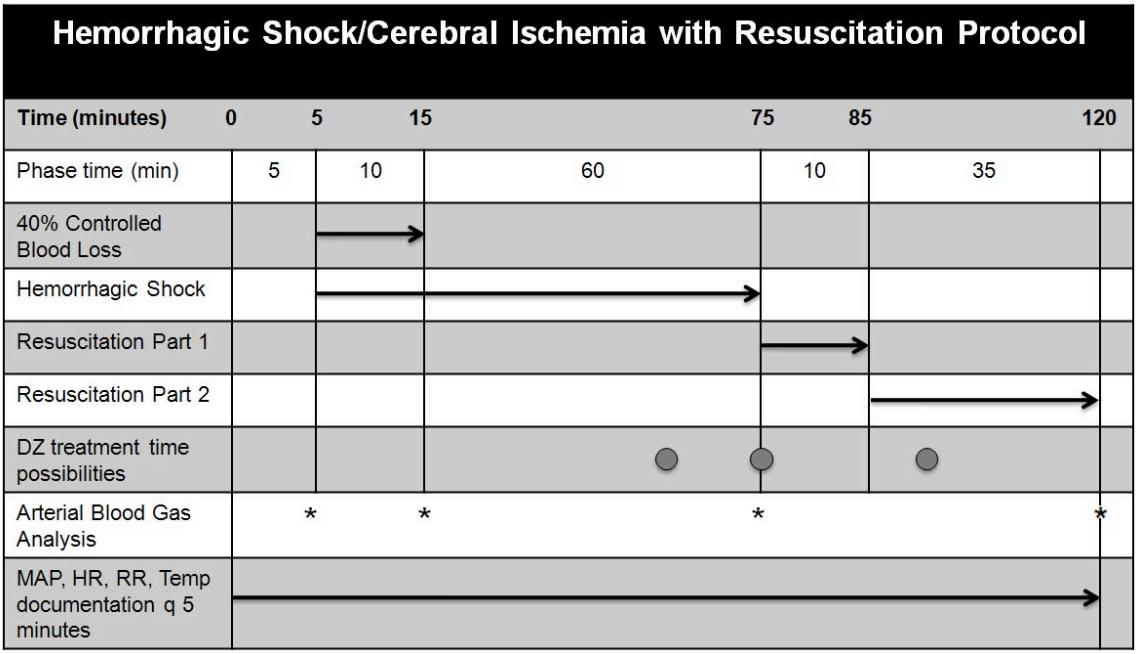


Table 1. Summary of Treatments for Groups

Groups	n Experiment 1	n Experiment 2	Treatment
Control	5	6	Animals received no surgical or experimental manipulations before euthanasia.
Sham	7	5	Anesthesia performed and an incision was made from the area of the mandibular salivary gland to the sternum. The right carotid artery (RCA) was exposed through blunt dissection.
UCAO	7	6	Anesthesia performed, the RCA was exposed and cannulated for arterial blood pressure and gasses (ABP and ABG).
UCAO VEH	7	6	Anesthesia performed, and the RCA was cannulated for ABP and ABG. The caudal vein was cannulated for peripheral IV access to allow the infusion of 1 ml of 0.9% NS vehicle.
UCAO DZ	7	6	Anesthesia performed, the RCA was cannulated for ABP and ABG. The caudal vein was cannulated for peripheral IV access for the infusion of 3.2 mg/kg of DZ in 1 ml 0.9% NS.
HSCH	10	7	Anesthesia performed, the RCA cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation of 0.9% NS given at 3 times the blood loss.
DZ40	10	9	Anesthesia performed and the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV 40 minutes following the hemorrhage of 40% BV (20 minutes prior to the resuscitation).
DZ60	10	8	Anesthesia performed and the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV at the onset of resuscitation.
DZ80	10	8	Anesthesia performed and the RCA was cannulated for ABP, ABG, and 40% blood volume removal over 10 minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV 20 minutes after the onset of resuscitation.
Total N	73	61	

Figure 2. HSP25 and HSP70 protein expression after shock and DZ treatments.

The relative protein levels of HSP25 and HSP70 were estimated from the optical densities of the corresponding protein bands after subtraction of background values obtained from the same lane. All samples were normalized to the amount of β-actin. The mean density of the Control group on each membrane was calculated and divided into each sample yielding ratio difference values, which are summarized for HSP25 and HSP70 in the top and bottom sets of graphs, respectively. When DZ was given 40 minutes following hemorrhage (20 minutes prior to resuscitation), HSPs were significantly elevated. The top four graphs show that HSP25 for the DZ40 group was significantly elevated in the right hippocampus ($p < 0.001$) and right cerebral cortex ($p=0.016$), while levels for all groups in the left cortex and hippocampus was not different. The bottom four graphs summarize HSP70 expression in the cortex and hippocampus. HSP70 for the DZ40 group was significantly elevated in the right hippocampus and right cerebral cortex ($p < 0.001$ for both). In the left cerebral cortex, the UCAO VEH group had elevated HSP70 ($p = 0.006$), the ratio change was small.

Figure 2. HSP25 and HSP70 protein expression after shock and DZ treatments.

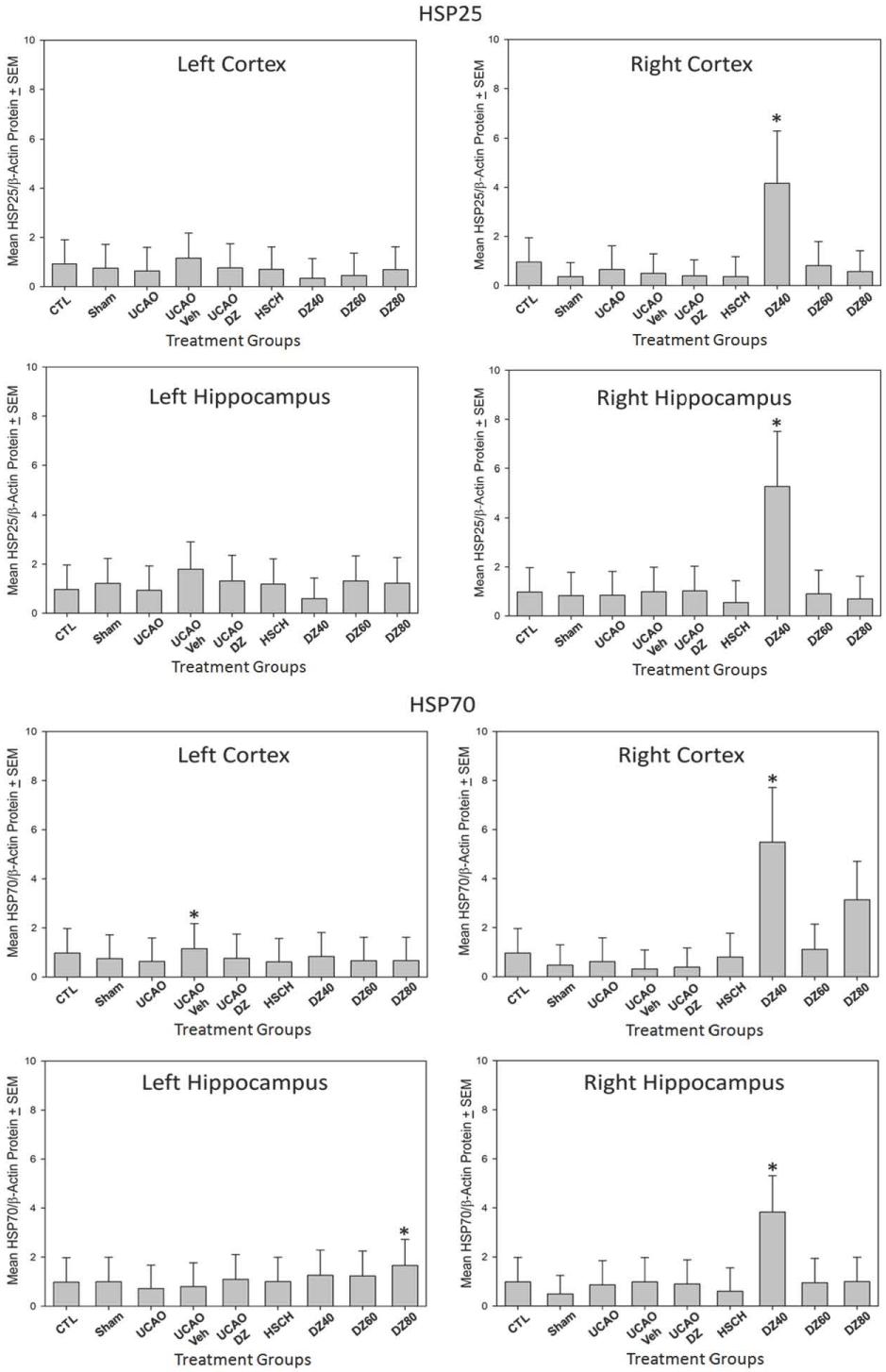


Figure 3. Western blots for HSP25 and HSP70.

Band density in brain tissue samples from the DZ40 animals was significantly greater in the right cortex (top two blots) and for HSP25 (bottom two blots), compared to all other treatment groups (all groups not illustrated).

Figure 3. Western blots for HSP25 and HSP70.

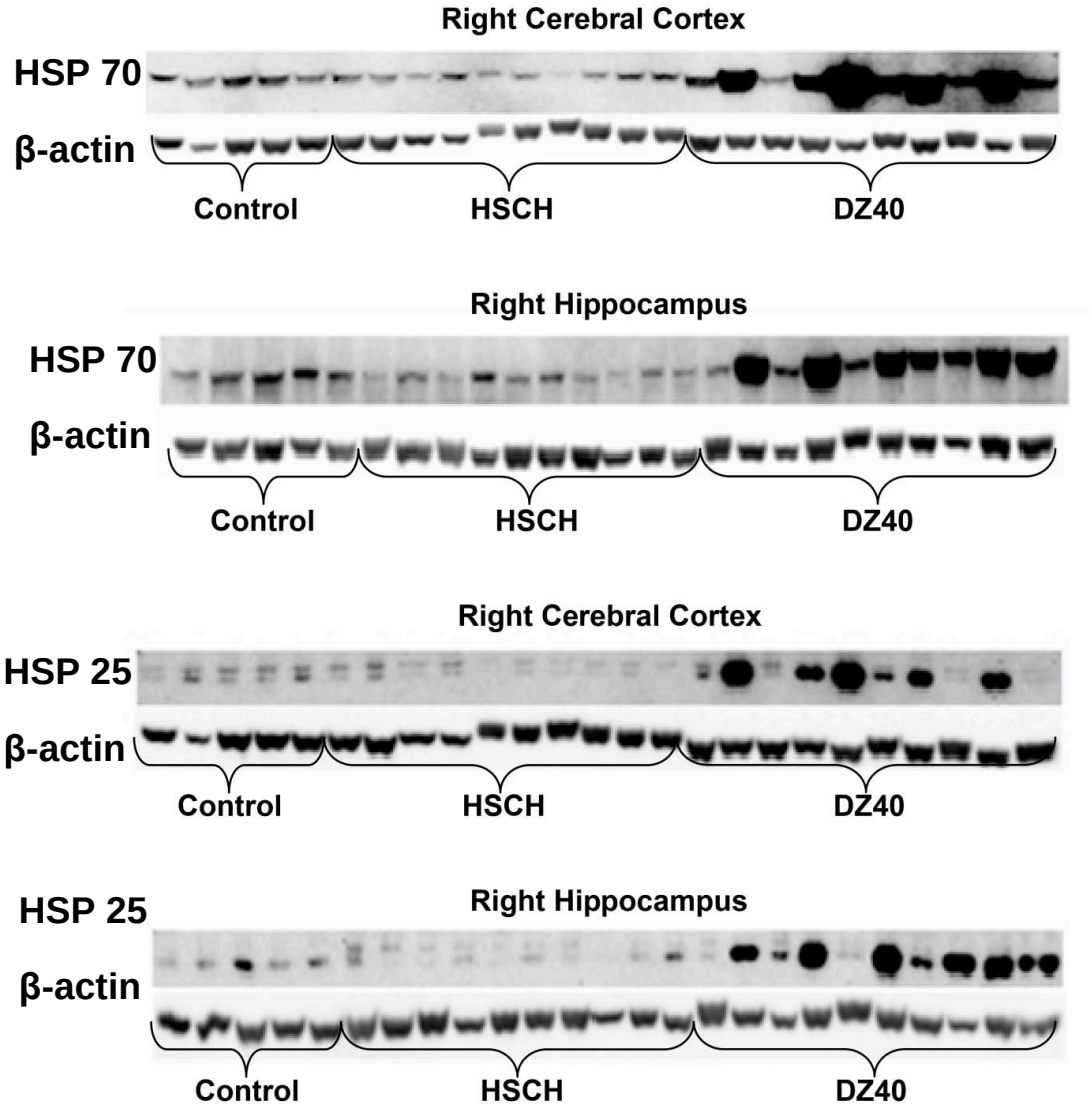


Figure 4. Locations of Observations

For immunohistochemistry analysis, observations were made under 50x magnification for the presence or absence of HSP staining and categorized as “1” for present or “0” for absent. Figure 4 represents the areas of the right and left cerebral cortices and hippocamppi that were observed with representative images below.

Figure 4. Locations of Observations

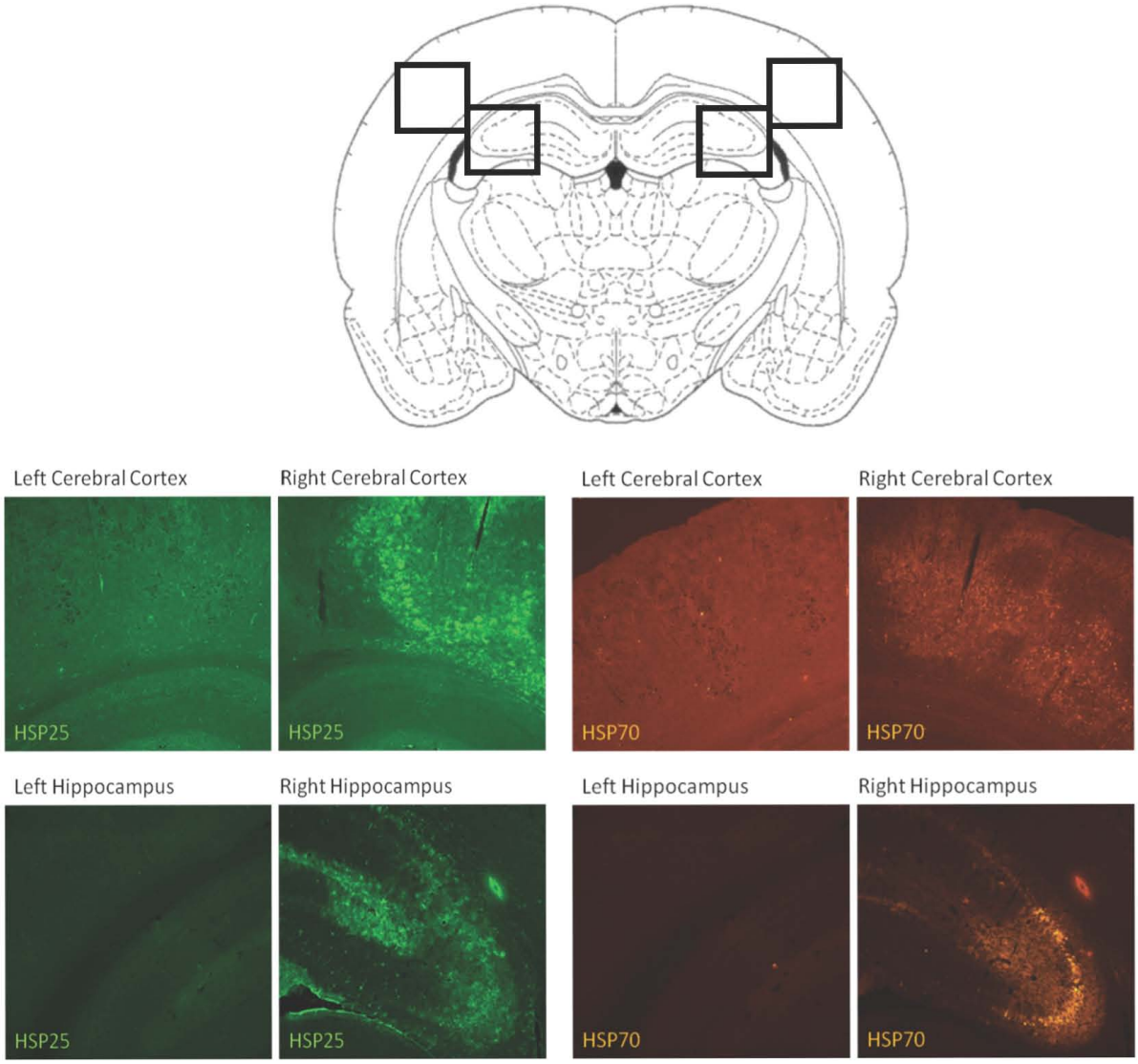


Table 2. Incidence of HSP25 Immunoreactivity in Right and Left Cerebral Cortices and Hippocamppi (RCC, RHIP, LCC and LHIP, respectively)

For immunohistochemistry analysis, observations were made under a 50x objective for the presence or absence of a HSP staining and categorized as “1” for present or “0” for absent and the group results were tallied. Fisher exact tests were performed to compare the number of animals of each group for relative staining for HSP expression. HSP25 activity was significantly elevated in the DZ40 group in the right cerebral cortex and hippocampus when compared to all other groups. These results echoed western blot data. The table summarizes the results from the Fisher exact tests for the presence or absence of immunostaining for HSP25. The annotations within certain groups correspond to the significance table located on the right.

Table 2. Incidence of HSP25 Immunoreactivity in Right and Left Cerebral Cortices and Hippocamppi (RCC, RHIP, LCC and LHIP, respectively)

Group	RCC	RHIP	LCC	LHIP
Control (n=5)	0	0	0	0
Sham (n=5)	0	0	0	0
UCAO (n=6)	0	1	0	0
UCAO VEH (n=5)	0	0	0	0
UCAO DZ (n=5)	0	0	0	1
HSCH (n=7)	1	2	0	0
DZ40 (n=9)	8 [1,2,3]	8 [4,5,6,7]	0	3
DZ60 (n=5)	0	0	1	0
DZ80 (n=6)	0	0	0	0

Significance Table		
Notation	Group x Group	P =
1	DZ40 x Control; Sham; UCAO VEH; UCAO DZ; DZ60	0.0029
2	DZ40 x UCAO; DZ80	0.0013
3	DZ40 x HSCH	0.0013
4	DZ40 x Control; Sham; UCAO VEH; UCAO DZ; DZ60	0.0029
5	DZ40 x UCAO	0.0109
6	DZ40 x HSCH	0. 0152
7	DZ40 x DZ80	0.0013

Table 3. Incidence of HSP70 Immunoreactivity in Right and Left Cerebral Cortices and Hippocamppi (RCC, RHIP, LCC and LHIP, respectively)

For immunohistochemistry analysis, observations were made under a 50x objective for the presence or absence of a HSP staining and categorized as “1” for present or “0” for absent and the group results were tallied. Fisher exact tests were performed to compare the number of animals of each group for relative staining for HSP expression. HSP70 activity was significantly elevated in the DZ40 group in the right cerebral cortex and hippocampus when compared to all other groups. These results echoed western blot data. The table summarizes the results from the Fisher exact tests for the presence or absence of immunostaining for HSP25. The annotations within certain groups correspond to the significance table located on the right.

Table 3. Incidence of HSP70 Immunoreactivity in Right and Left Cerebral Cortices and Hippocamppi (RCC, RHIP, LCC and LHIP, respectively)

Group	RCC	RHIP	LCC	LHIP
Control (n=5)	0	0	0	0
Sham (n=5)	0	0	0	0
UCAO (n=6)	0	1	0	0
UCAO VEH (n=5)	0	0	0	0
UCAO DZ (n=5)	0	0	0	1
HSCH (n=7)	1	2	0	0
DZ40 (n=9)	8 [1,2,3]	8 [4,5,6,7,8,9]	0	3
DZ60 (n=5)	0	0	1	0
DZ80 (n=6)	0	0	0	0

Significance Table		
Notation	Group x Group	P =
1	DZ40 x Control; Sham; UCAO VEH; UCAO DZ; DZ60	0.0029
2	DZ40 x UCAO; DZ80	0.0013
3	DZ40 x HSCH	0.0013
4	DZ40 x Control; Sham; UCAO VEH; UCAO DZ; DZ60	0.0029
5	DZ40 x UCAO	0.0109
6	DZ40 x HSCH	0. 0152
7	DZ40 x DZ80	0.0013

Figure 5. Characteristic Patterns of HSP25 and HSP70 Expression

This figure represents the right cerebral cortex (top left), right hippocampus (top right), right thalamus (bottom left), right hypothalamus (bottom center), and the amygdala/piriform cortex (bottom right). The yellow scale bars in the top right of each picture represent a 20 micrometer distance. HSP25 (green) appeared to be present mostly in astrocytic-like cells while HSP70 (red) was present in neuronal-like cell profiles. HSP25 in many regions surrounded HSP70 and it has been suggested that HSP70 neuronal staining is indicative of vulnerable cells that will survive the ischemic insult that border the infarcted zone of injury. An intense colocalization of HSP25 and HSP70 was consistently observed in the CA2, CA2/3, and CA3 region of the right hippocampus. Colocalization was also found within the right thalamus to a great extent. Finally, HSP25 and HSP70 were observed in the hypothalamus, amygdala and piriform cortex.

Figure 5. Characteristic Patterns of HSP25 and HSP70 Expression

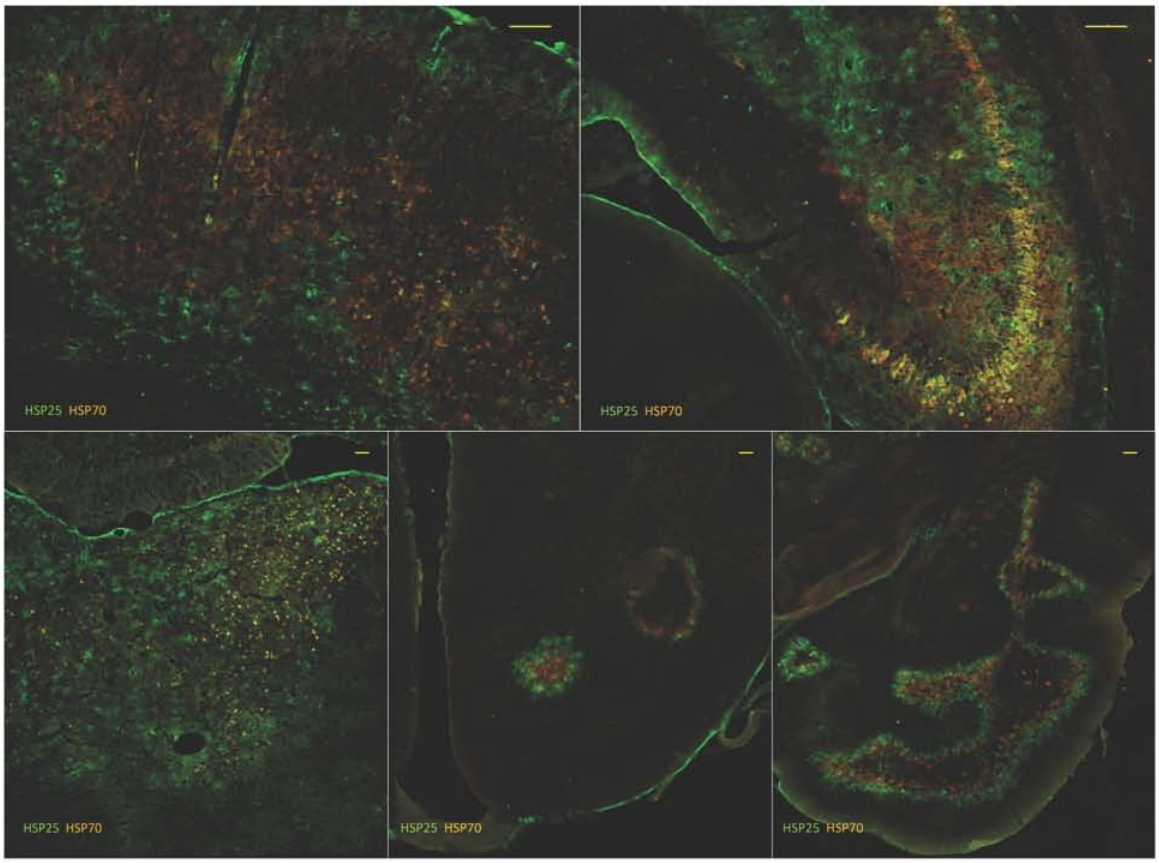
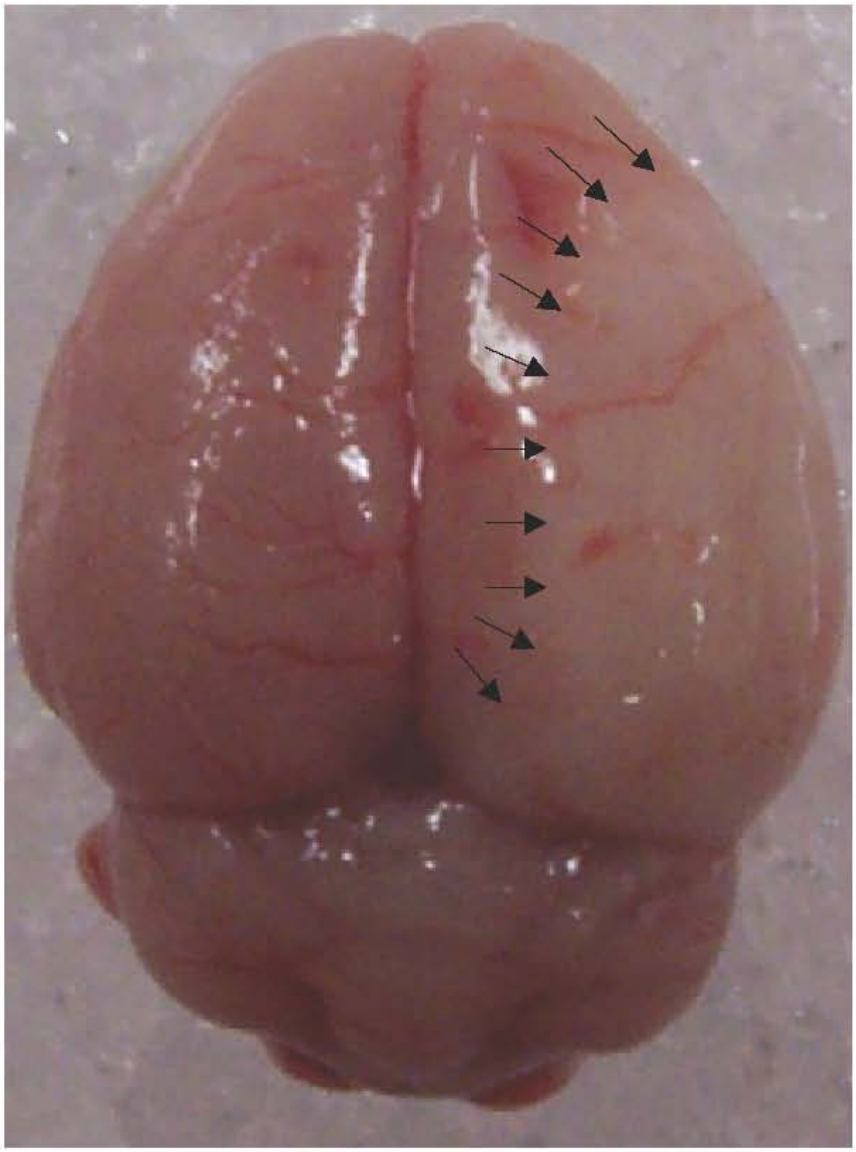


Figure 6. Representation of Right Hemispheric Hemorrhagic Injury.

Figure 7 represents a typical injury pattern observed following implementation of the hemorrhagic shock model. The arrows delineate a pale region of hypovascular, edematous cortex.

Figure 6. Representation of Right Hemispheric Hemorrhagic Injury.



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Chapter 4

RESUSCITATION WITH DIAZOXIDE DECREASES HEMORRHAGIC SHOCK-INDUCED BRAIN LEVELS OF CLEAVED CASPASE-3

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Abstract

Hemorrhagic shock is the leading cause of death following an accidental or combat-related trauma. Worldwide, over 5 million people died from trauma-related injury in 2000, and up to 64% of those fatalities suffered a coinciding brain injury. For nearly a century, researchers have investigated resuscitative strategies to better the outcomes of traumatic casualty treatments. The initial measures in management, hemostasis and volume restoration are vital but too often prove unsuccessful in improving morbidity and mortality. As a result, our team investigated the hypothesis that diazoxide (DZ), a mitochondrial K_{ATP} channel opener, could be used during resuscitation to induce a postconditioning effect in a laboratory animal model of hemorrhagic shock with concomitant cerebral ischemic injury. Male Sprague-Dawley rats underwent a 40% plasma volume hemorrhage and resuscitation one hour later. DZ was administered at one of three time points: 20 minutes before the onset of resuscitation, at the time that resuscitation began, or 20 minutes after the initiation of resuscitation. Immunohistochemistry techniques were used to assess the ability of DZ to decrease cleaved caspase-3, a regulator of apoptosis, within the cerebral cortex and hippocampus following injury. The administration of DZ in the initial resuscitation volume significantly reduced caspase-3 activity bilaterally in regions of the hippocampus and the perirhinal cortex following hemorrhagic shock.

Hemorrhagic shock is the leading cause of death following an accidental or combat-related trauma [1, 2]. Worldwide, over 5 million people died from trauma-related injury in 2000, and up to 64% of those fatalities suffered a coinciding brain injury [3]. Following exsanguinating trauma, hemorrhagic shock will result when cardiac output is no longer capable of maintaining blood flow and delivering the oxygen necessary to meet organ metabolic demands. The brain, as a result of the inability to store glucose, requires a steady-state of oxygen delivery to meet its metabolic requirements. When mean arterial pressure drops below 60 mmHg, cerebral perfusion pressure begins to decline, and as pressure falls below 25-35 mmHg neuronal cell death ensues [4]. To add further insult, when blood loss approaches 40%, there is a 160% increase of oxygen consumption [5]. Taken together, the decline in oxygen delivery coupled with an increase in oxygen consumption sets the stage for global cerebral injury. This type of indirect cerebral trauma has been shown in humans suffering a hemorrhagic injury [6].

Resuscitation for hemorrhagic injury—while crucial—is not benign. The mechanisms involved with reperfusion injury are complex and include the rapid and persistent generation of reactive oxygen species, increases in cytosolic and mitochondrial calcium levels, and the opening of the mitochondrial Permeability Transition Pore [7]. Following prolonged ischemia, these mechanisms give rise to areas of irreversible cell death and regions of vulnerable cells. Restoration of flow associated with resuscitation results in organ recovery but can incompatibly extend the area of irreversible cell death.

Fluid resuscitation choices are still a topic of debate, but two considerations are clear: 1) current resuscitative protocols do not address neuroprotective strategies to reduce primary cerebral ischemic injury and attenuate secondary reperfusion injury following hemorrhagic shock [2, 8, 9] and 2) options that promote prosurvival phenotypes are the next step in resuscitation research [10].

One means for promoting survival following injury is the phenomenon known as postconditioning. Postconditioning, which had its origin in cardiac physiology research [11, 12], is typically employed through cycles of blood vessel release and re-occlusion. These cycles follow a period of prolonged vessel occlusion resulting in ischemia but prior to establishing continual organ reperfusion. This mechanical maneuver significantly decreases cardiac tissue injury resulting from ischemia-reperfusion and can be mimicked in the heart pharmacologically with mitochondrial potassium ATP-sensitive (mK_{ATP}) channel openers given before and during the reperfusion period [13].

It is evident that severe hemorrhage can also generate ischemic brain injury and that reperfusion injury may compound the insult. However, there has been scant research for strategies into protective strategies for resuscitating the brain following hemorrhagic injury. Studies have found apoptotic mechanisms in the ischemic brain, including the extrusion of cytochrome c from mitochondria to cytosol, eventually resulting in the cleavage and activation of caspase-3, and the mechanisms of reperfusion injury can exacerbate these processes [14-17]. Recently, our laboratory has found that diazoxide (DZ), a mK_{ATP} channel opener,

given following hemorrhagic shock upregulates the protective heat shock proteins (HSP) 25 and 70 within the brain [18, 19], proteins which have been found to directly inhibit caspase-3 activity [20, 21]. Additionally, DZ has been found to decrease ischemia-reperfusion injury and caspase-3 activity in rabbit spinal cords [22]. Here, our laboratory tested the hypothesis that DZ, used as part of a resuscitative protocol (Figure 1) following hemorrhagic shock, could serve as a postconditioning trigger and attenuate cerebral cellular injury as identified by cleaved caspase-3 (CC3) activity.

To test this hypothesis, a postconditioning paradigm was employed where DZ was given at three different time points in a model of hemorrhagic shock with concomitant cerebral ischemic injury ([19]; Figure 1). Fifty-one male Sprague-Dawley rats (290–375 grams) from Charles River Laboratories (Wilmington, MA) were quartered for 48-72 hours with a 12-hour light/dark cycle, constant temperature (24°C), and given unlimited access to water and rat chow. All procedures were approved by the Institutional Animal Care and Use Committee of the Uniformed Services University of the Health Sciences, Bethesda, Maryland.

Prior to surgery, rats were randomly assigned to one of nine groups (Table 1). Briefly, anesthesia was induced with Isoflurane (Abbott Labs, Chicago) at 5% concentration in room air until loss of response to tail clamp. Following induction, the right carotid artery was cannulated for arterial blood pressure (ABP) monitoring and hemorrhage while the tail vein was cannulated for intravenous (IV) therapy. Body temperature was monitored real-time and strictly kept

between 36.5–37.5^{o}C using a radiant heat lamp and rectal probe. Following a 5-minute baseline recording of vital signs, animals in Groups 6-9 (Table 1) received a controlled hemorrhage of 40% blood volume over a ten minute period. Blood volume was calculated with the following equation: BV in ml = (0.06 X body weight in grams) + 0.77 [23]. Mean arterial pressures initially declined to 20-30 mmHg in shocked groups (data not shown) and rats remained in a shocked, non-resuscitated state for 60 minutes.

DZ (Sigma, St. Louis) was prepared by dissolving 10mg of DZ in 1ml of 0.5μM NaOH then diluted in 10 ml of normal saline (NS) for a final concentration of 1mg/ml. DZ 3.2 mg/kg was given intravenously at one of three time points (Figure 1). Sixty minutes following shock, the first phase of resuscitation was initiated. In the first ten minutes of resuscitation, rats received 0.9% NS based upon the following formula: first ten minutes resuscitation volume (FTMRV) = body weight in kilograms X 28.57ml/kg. FTMRV mimics the administration of 2 liters of lactated Ringer's solution based upon an average 70kg male human as recommended by the ATLS guidelines following hemorrhagic traumatic injury [8]. Following FTMRV, the second phase of resuscitation consisted of pro-rating the remainder of resuscitation volume over the last 35 minutes of procedural time so that the total resuscitation volume equaled three times the amount of blood lost. Following surgery and recovery, animals were returned to their home cages.

Twenty-four hours following surgery, rats were anesthetized with intraperitoneal injections of 80mg/kg Ketamine (Fort Dodge Animal Health, Fort

Dodge, IA) and 10mg/kg Xylazine (Phoenix, St. Louis). Rats were transcardially perfused with 100mM phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Whole brains were retrieved and placed in 4% paraformaldehyde in PBS overnight at 4^{o}C. The following day the brains were transferred into 30% sucrose for 1-3 days and then stored at -80^{o}C. Coronal sections (20µm) were retrieved using a cryostat, placed in cryoprotectant and transferred to storage at –20^{o}C. Sections in the area of coordinates interaural 5.76 mm, bregma -3.24 mm [24] were free-floated onto gelatin-chromium potassium sulfate submersed slides. Slides were dried and then stored at –20^{o}C. Following slide fixation with 10% formalin for 30 minutes, antigen retrieval processing took place where slides were heated in Tris-EDTA Buffer (10 mM Tris Base, 1 mM EDTA, 0.05% Tween 20, pH 9.0) at 100^{o}C for 10 minutes and washed in water for 20 minutes. After blocking for one hour at room temperature, slides were incubated with primary antibodies (CC3 #9661 1:50, Cell Signaling, Danvers, MA) for 2 hours at 37^{o}C followed by incubation for 1 hour at 37^{o}C with species appropriate fluorescently-conjugated secondary antibodies.

Following mounting, images of selected regions of the cortex and hippocampus (Figure 2) were obtained using 20x magnification with an Axioplan inverted microscope coupled with an Axiovision camera and software (Zeiss, Thornwood, NY). Brain tissue 20x images were converted to black and white images and observed for CC3 staining. CC3 was manually counted using the ImageJ cell counter application. Separate ANOVAs were conducted for each

brain region data but if normality or variance tests failed, the Kruskal-Wallis ANOVA was performed.

In four brain regions of tissue samples from the DZ60 group there was a significant overall difference between the nine treatment groups, but *post hoc* comparisons to the HSCH group indicated no differences (Table 2). However, in five of the remaining 12 brain regions of animals receiving DZ at the time of resuscitation (DZ60 group), counts of the number of CC3-positive cells profiles was significantly less in the DZ60 samples than in the HSCH samples (Table 2). These regions included the right CA2 region, CA3 region, and the perirhinal cortex as well as the left CA2/3 region and perirhinal cortex (Figure 2). The only other group showing areas of significantly decreased CC3 counts was the UCAO VEH group. In this *non-hemorrhagic shock group*, the CA1, CA2/3, and cerebral cortex on the *un-occluded* left side were lower (Figure 2). Although not significantly different, CC3 cell counts were also lower in other brain regions of the DZ60 group, as well as in the DZ40 and DZ80 groups. The latter may suggest a larger sample size would have found treatment at these time points after shock also reduce CC3 cell counts.

For nearly a century, researchers have investigated resuscitative strategies to better the outcomes of traumatic casualty treatments. The initial measures in management, hemostasis and volume restoration are vital but too often prove unsuccessful in improving morbidity and mortality. The present study indicates the addition of DZ in the initial resuscitation volume (the DZ60 group) significantly reduced caspase-3 activity bilaterally in regions of the hippocampus

and the perirhinal cortex following hemorrhagic shock. This may be clinically significant for morbidity since caspase-3 has been reported to play a major role in apoptosis. As reviewed by Porter and Janicky [25], once caspase-3 is activated, downstream death substrates are cleaved and cleaved caspase-3 may amplify the upstream death cascade to include cytochrome c release.

The mechanism as to why opening of the mK_{ATP} channel at the onset of resuscitation decreased caspase-3 activation is unknown. Previous work in our laboratory has found postconditioning with DZ in hemorrhagic shock upregulates HSP25 and HSP70 within the brain [18, 19], proteins which have been found to directly inhibit caspase-3 activity [20, 21]. Additionally, Akao and colleagues found that DZ preserved mitochondrial integrity and decreased mitochondrial depolarization in oxidatively-stressed cardiomyocytes when given at 100 µmol/L concentration [26]. Calculations estimated plasma concentrations in this investigation may range from 200 to 220 µmol/L. In 2003, this group replicated this result twice in cerebellar granule neurons that underwent oxidative stress. By preserving mitochondrial integrity, cytochrome C release was reduced. In these studies, 100 DZ or 100 µmol/L nicorandil decreased caspase-3 activation by 40% at sixteen minutes following the onset of stress [27, 28].

To our knowledge, this is the first evidence that opening the mK_{ATP} channel using DZ at the onset of resuscitation decreases caspase-3 activity. Although further investigation is necessary to decipher the mechanisms through which DZ reduced cleaved caspase-3 levels, and further work is warranted to determine if there is a concomitant decrease in apoptotic injury, postconditioning

with DZ may offer a new strategy in the treatment of hemorrhagic shock. Resuscitative protocols currently do not address measures to promote cell survival. DZ, an agent used safely in humans for decades, may offer a novel therapy in preventing brain ischemia-reperfusion injury.

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Table 1: Summary of Groups

This table lists the group name and number designation, the sample size for each group and the experimental treatment each group underwent.

Table 1: Summary of Group Treatments

Groups	n	Treatment
Control	**5**	The animals received no anesthetic, surgical or experimental manipulations prior to euthanasia.
Sham	**5**	Anesthesia performed and an incision was made from the area of the mandibular salivary gland to the sternum. The right carotid artery (RCA) was exposed through blunt dissection.
UCAO	**3**	Anesthesia performed, the RCA was exposed and cannulated for arterial blood pressure (ABP).
UCAO VEH	**5**	Anesthesia performed, and the RCA was cannulated for ABP. The caudal vein was cannulated for peripheral IV access to allow the infusion of 1 ml of 0.9% NS vehicle.
UCAO DZ	**5**	Anesthesia performed, the RCA was cannulated for ABP. The caudal vein was cannulated for peripheral IV access for the infusion of 3.2 mg/kg of DZ in 0.9% NS.
HSCH	**5**	Anesthesia performed, the RCA cannulated for ABP and 40% blood volume removal over ten minutes. The caudal vein was cannulated for peripheral IV access and resuscitation.
DZ40	**7**	Anesthesia performed and the RCA was cannulated for ABP and 40% blood volume removal over ten minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg in 0.9% NS IV 40 minutes following the hemorrhage of 40% BV but 20 minutes prior to the resuscitation.
DZ60	**8**	Anesthesia performed and the RCA was cannulated for ABP and 40% blood volume removal over ten minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg IV mixed into the FTMRV at the onset of resuscitation.
DZ80	**8**	Anesthesia performed and the RCA was cannulated for ABP and 40% blood volume removal over ten minutes. The caudal vein was cannulated for peripheral IV access and resuscitation. Received DZ 3.2 mg/kg in 0.9% NS IV 20 minutes after the onset of resuscitation.
Total N	**51**	

Figure 1. Hemorrhagic shock with concomitant cerebral injury protocol.

Animals were anesthetized and monitors/lines placed. Baseline readings of vital signs (heart rate, respirations, temperature, mean arterial pressure) were documented and then induction of 40% controlled blood loss occurred. Following hemorrhage, the animal was left in a shocked state for 60 minutes. After 60 minutes, the 1st part of resuscitation, the first ten minutes resuscitation volume (FTMRV), occurs as the animal received 28.57 ml/kg of 0.9% NS. The animal then received 0.9% NS over the next 35 minutes and total fluid resuscitation was three times the amount of blood loss during hemorrhage. Diazoxide (3.2 mg/kg IV) treatment occured in certain groups at one of three time points (indicated by filled circles), either 40 minutes following hemorrhage (DZ40), at the time of the start of resuscitation (DZ60), or 20 minutes after resuscitation starts (DZ80). Vital signs were documented every 5 minutes. Isoflurane-indueced anesthesia was maintained as follows: 1.0% for the first 5 minutes, 0.6% during hemorrhage, 1.0% during the remainder of the surgical procedure. Arterial blood gases were drawn at four time points: at the onset of hemorrhage, at the end of hemorrhage, at the beginning of resuscitation, and at the end of the surgical procedure (indicated by asterisks in figure).

Figure 1. Hemorrhagic shock with concomitant cerebral injury protocol

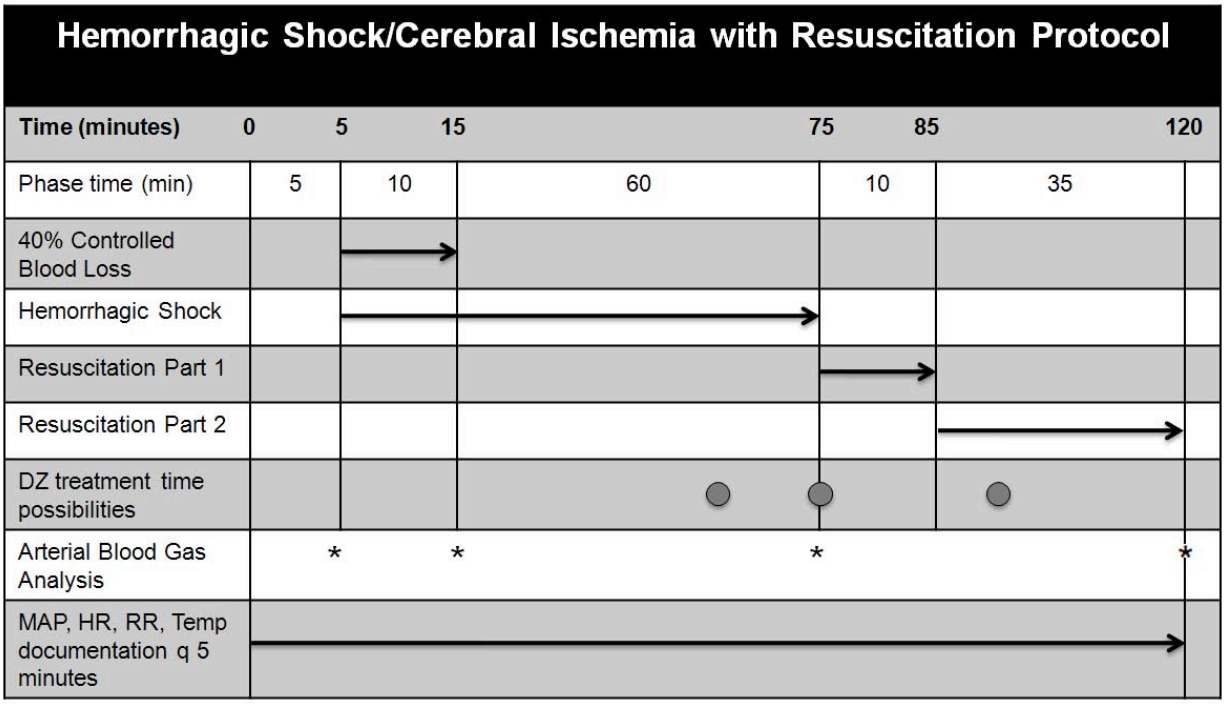

Figure 2: CC3 Immunostaining Compared to the HSCH Group

Images of selected regions of the cortex and hippocampus were obtained using a 20x objective. Images were converted to black and white and CC3 counts were performed. Figure 2 shows the regions with significantly decreased CC3 activity when the DZ60 group was compared to the HSCH group (top). In four brain regions of tissue samples from the DZ60 group there was a significant overall difference between groups, but post hoc comparisons to the HSCH group indicated no statistically significant differences. However, in the right CA2, right CA3, right perirhinal cortex, left CA2/3, and left perirhinal cortex, the DZ60 group CC3 counts were significantly less than in the HSCH samples. The only other group showing areas of significantly decreased CC3 counts was the UCAO Veh group (bottom). In this non-hemorrhagic shock group, the CA1, CA2/3, and cerebral cortex on the un-occluded left side were lower.

Legend for Brain Regions:

L or R	left or right
CA1	CA1 region of hippocampus
CA2	CA2 region of hippocampus
CA2/3	CA2/3 region of hippocampus
CA3	CA3 region of hippocampus
CC	cerebral cortex
PC	perirhinal cortex

Figure 2: CC3 Immunostaining Compared to the HSCH Group

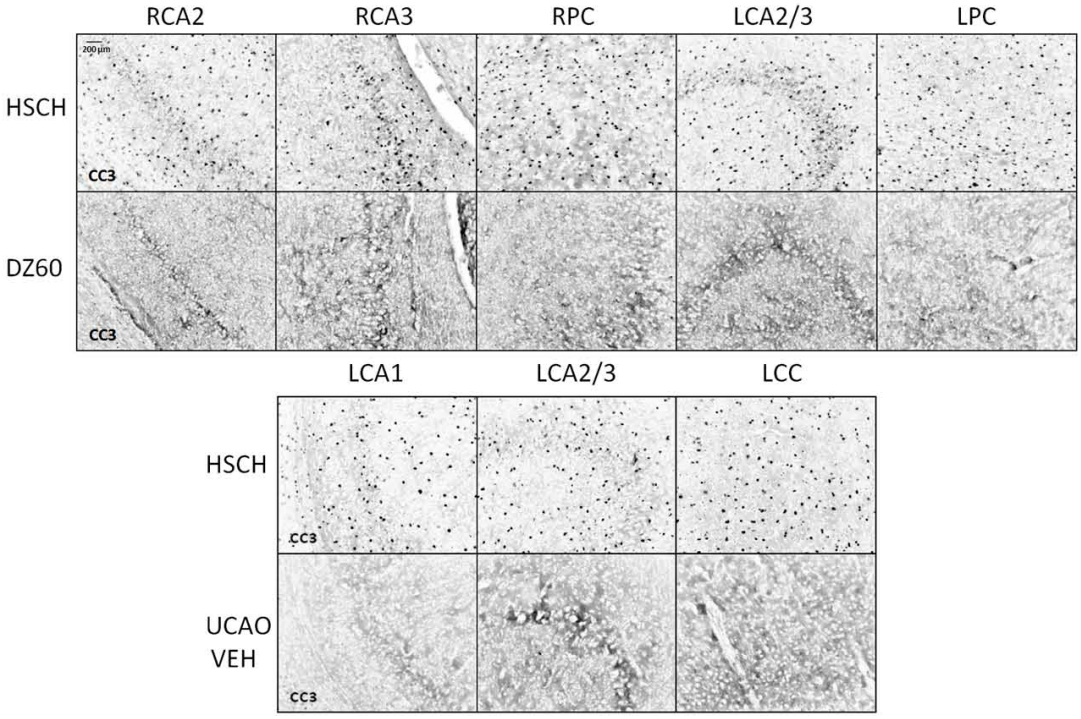

Table 2. Summary of cleaved caspase-3 (CC3) immunostaining after Unilateral Common Carotid Artery Occlusion (UCAO), Hemorrhagic Shock (HSCH) and treatments with Diazoxide (DZ).

Brain regions were observed using a 20x objective and CC3 counts were manually tallied using the ImageJ cell counter application. The cell counts are listed on top with the standard deviation in parentheses below the cell count. All groups were compared the HSCH group (as annotated by the asterisk). Numbered superscript annotations in certain brain regions correspond to significant *post hoc* p values listed in the box located on the right when compared to the HSCH group.

Table 2. Summary of Cleaved Caspase-3 Immunostaining after Unilateral Common Carotid Artery Occlusion (UCAO), Hemorrhagic Shock (HSCH) and Treatments with Diazoxide (DZ)

	Treatment Group								
	Control	Sham	UCAO	UCAO Veh	UCAO DZ	HSCH*	DZ40	DZ60	DZ80
Brain Region									
RCA1	147.4 (30.96)	64.6 (29.54)	194 (23.07)	111.6 (28.36)	178.6 (16.99)	146.6 (19.81)	83.71 (26.18)	66.75 (10.29)	97.5 (18.13)
RCA2	155 (28.45)	71 (31.89)	179.6 (23.56)	118.2 (30.18)	176.6 (16.71)	152 (23.68)	90 (28.19)	54.37[1] (8.24)	80.8 (17.54)
RCA2/3	157 (36.79)	63.2 (32.06)	169.33 (19.12)	100.6 (30.44)	168.2 (22.81)	115 (27.55)	93 (25.08)	49.12 (10.62)	72.87 (16.94)
RCA3	138.8 (31.17)	59.4 (29.04)	173.3 (11.31)	102.6 (32.12)	167.4 (9.18)	148.2 (31.24)	99.33 (27.14)	43.5[2] (7.86)	72 (17.54)
RCC	90.4 (18.50)	58.2 (21.84)	108.6 (20.85)	88.4 (37.56)	101.2 (13.54)	114.5 (26.47)	94.71 (19.38)	60.37 (9.85)	81.87 (9.85)
RPC	128 (6.34)	98 (14.81)	132 (44.24)	91.6 (20.51)	159 (7.11)	161.4 (22.53)	98.85 (29.91)	59[3] (17.50)	85 (10.99)
LCA1	156.2 (15.85)	52.2 (21.28)	141.6 (35.57)	42.6[6] (19.78)	169.2 (6.39)	178 (29.58)	119.4 (36.39)	53.37 (12.54)	104.7 (18.12)
LCA2	146 (20.27)	45.8 (18.64)	140.6 (45.67)	49 (35.61)	171.8 (5.24)	161.2 (26.59)	124.8 (40.69)	50.6 (12.04)	98.7 (19.35)
LCA2/3	153.8 (22.77)	67.2 (41.16)	131.6 (27.48)	56[7] (39.72)	144.2 (9.63)	173.4 (20.44)	107.1 (38.20)	41.1[4] (9.30)	93.8 (17.51)
LCA3	143.2 (13.96)	69.2 (36.79)	121.6 (49.40)	53[8] (36.15)	159.6 (14.85)	160.4 (22.32)	115.4 (35.83)	43.2 (9.00)	93.7 (17.69)
LCC	103.8 (13.92)	39.2 (12.80)	102.6 (24.22)	31.4 (22.44)	94.6 (24.36)	117.4 (21.66)	67.2 (24.09)	59.6 (15.08)	62.6 (9.97)
LPC	130.4 (12.46)	100 (24.26)	135.6 (38.30)	85.6 (32.99)	152.4 (12.67)	149 (21.80)	94.7 (26.18)	54.4[5] (14.82)	81 (12.56)

1	p<0.001
2	p<0.001
3	p<0.013
4	p<0.011
5	p<0.041
6	p<0.001
7	p<0.011
8	p<0.042

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Chapter 5

DISCUSSION

Objective

The objective of this work was to evaluate the potential neuroprotective effect of DZ when used as part of a resuscitative protocol following hemorrhagic shock and concomitant cerebral ischemic injury. Previous investigations have documented the protective effects of DZ and other mK_{ATP} channel agonists in other brain postconditioning ischemic-reperfusion paradigms typically dealing with stroke-induced injury. A review of the literature, however, indicates that our team is the only group to investigate the utility of mK_{ATP} channel agonists in this capacity [1, 2].

The clinical utility of postconditioning following hemorrhage is significant as potentially protective pharmacological interventions can be employed *after* a traumatic injury, which could offer a defense against both the primary ischemic insult and secondary reperfusion injury. The impact trauma has on the global population and medical system is well documented. It is estimated that nearly 0.1% of the world's population died from traumatic injury in the year 2000 accounting for 5 million people [3]. In addition, it is estimated that 40% of trauma-related deaths are a result of hemorrhage [4]. As for the economic burden of trauma, in 2000 in the United States alone, almost 1% of its Gross Domestic Product, 117 billion dollars, was spent for treating trauma related injuries [5]. This investigation provides a framework which can be used to

address two critical but missing components of trauma resuscitation research: 1) interventions to protect the brain, and 2) mechanisms that confer cytoprotection when employed following trauma-induced hemorrhage.

Post-hemorrhagic pre-resuscitation conditioning using DZ increases HSP25 and HSP70 in the cerebral cortex and hippocampus (Figure 1).

The aim of this experiment was to assess the ability of DZ postconditioning at different time points to express HSP25 and HSP70 within the brain subsequent to hemorrhagic shock. Only one other study has investigated DZ used in this capacity [2]. In that study, O'Sullivan and colleagues suggested that postconditioning with DZ may increase HSP25 and HSP70 within the cerebral cortex and hippocampus when given immediately prior to resuscitation. O'Sullivan's results were limited to western blotting assays and utilized a model of hemorrhagic shock that involved extensive trauma to the rat's right lower extremity. This model, while useful, may have confounded the results of the investigation as it has been determined that brain injury can be greatly exacerbated when there is a coinciding traumatic injury [6]. To reduce the poly-traumatic nature of the preparation, the model used by O'Sullivan was modified as discussed in Chapter 3 and involved no extremity injury. A consequence that was immediately evident was that mortality rates improved significantly so that nearly 95% of all animals who underwent shock survived. This use of the common carotid artery as both a source for exanguination and comcomitant

occlusion, in contrast to femoral artery and vein occlusion, may in part have played a role in our differing mortality rates.

Our team gave DZ at one of three points after hemorrhagic shock: 20 minutes prior to, concomitant with, or 20 minutes after the onset of resuscitation. As stated in Chapter 3, these time points were selected based upon the average evacuation times found in military and civilian trauma settings [7, 8]. Western blot results indicated that HSP25 and HSP70 were both highly expressed when DZ was administered 20 minutes prior to the onset of resuscitation. When DZ was given either concomitantly with or 20 minutes after resuscitation, HSP levels did not show any difference from controls. Additionally, animals in the HSCH group did not show any change in HSP25 or HSP70 levels.

The western blot results were corroborated by immunohistochemistry methods. Again, the administration of DZ resulted in a robust expression of HSP25 and HSP70 in the DZ40 group. This activity was by and large absent in all other groups. When observing patterns of expression, HSP25 with an astrocytic profile consistently surrounded neuronal-like HSP70 expression and some areas. In the right CA2/3 region in what appeared to be pyramidal neurons and in the thalamus, however, there was colocalized staining for both HSPs. This colocalization data has not been reported in the literature previously. It has been discovered that glial-induced HSP27, the primate analogue of HSP25, localizes at the synaptic connections of neurons [9]. One possibility that could explain the robust colocalization activity is a transfer of HSP25 produced in glial

to neuronal cells with extracellular receptor binding of HSP25 and subsequent neuronal internalization [10, 11]. If true, this would not contradict several studies suggesting glial and neuronal specificity for HSP25 and HSP70, respectively, in ischemic paradigms [12-18].

These results suggest that optimal DZ administration following hemorrhagic shock may be during the ischemic period well in advance of baseline restoration of blood flow (Figure 1). When given intravenously DZ has an onset of action of 1-2 minutes when given intravenously [19], this may be extended with a decreased cardiac output. Determining the stringency of the timing is the focus of further research, but these results are clear. Post-hemorrhagic pre-resuscitation conditioning with DZ greatly increases HSP25 and HSP70 in the rat cerebral cortex and hippocampus ipsilateral to the injury in western blot and immunohistochemistry analysis.

Resuscitation with DZ decreases hemorrhagic shock-induced brain levels of cleaved caspase-3 (Figure 2).

The aim of this experiment was to assess the ability of DZ postconditioning, given at different time points subsequent to hemorrhagic shock, to alter GFAP and cleaved caspase-3 levels. When reviewing the literature, no other study of this type has been conducted. As previously described, DZ was given at three time points following hemorrhagic shock; before, at the time of, and after the initiation of fluid resuscitation.

Among the groups, there was no difference found in GFAP levels. As well, hemorrhagic shock, which was expected to increase GFAP, showed no effect. GFAP was selected as a marker of injury and as reviewed in Eng, Ghirnikar, and Lee [20], reactive astrogliosis historically has been a prominent feature of astrocytes adjacent to injury. In addition, ischemic preconditioning reduced GFAP levels after retinal ischemia in rats [21]. More recently, GFAP levels were reduced by postconditioning in a retinal ischemic model [22]. These results could possibly be a result of timing. We did not measure mRNA but Burtrum and Silverstein [23] found after 24 hours in a rat neonatal hypoxic-ischemic paradigm, that GFAP mRNA was significantly elevated. Also, in a rat MCAO model of ischemia, Whitehead and colleagues [24] showed that GFAP protein was elevated three days after injury. It is possible that the assessment of GFAP protein 24 hours after ischemia was too early to detect a difference.

During our research,we became aware that HSP25 and HSP70 have both been found to inhibit the cleavage of caspase-3 [25, 26]. In addition, the ischemic brain displays apoptotic activity with the mitochondrial release of cytochrome C and resultant cleavage of caspase-3. As well, reperfusion exacerbates this process [27-30]. As a result, we assessed cleaved caspase-3 activity through immunohistochemistry. The data suggested that giving DZ concomitantly with resuscitation and mixed within the initial resuscitation volume significantly decreased cleaved caspase-3 activity bilaterally in the hippocampus and piriform cortex when compared to the HSCH group. Although HSP25 and HSP70 have been found to inhibit caspase-3 cleavage, the DZ60 group did not

have elevated HSP levels in the first experiment (Chapter 3). Caspase-3 cleavage and depolarization of the mitochondrial inner membrane are very rapid and occur in parallel and once activated, caspases are held in check by sequestration by heat shock chaperone (HSC) 70, thereby depleting the free pool of HSC70, leading to increased synthesis of HSP70 [31, 32]. This may explain why HSPs were elevated in the DZ40 group but showed no difference in caspase-3 cleavage. As for the DZ60 group, the mechanism by which DZ may attenuate caspase-3 cleavage at the time of resuscitation is unknown. When DZ is introduced simultaneous to a reoxygenated ischemic area it may better preserve mitochondrial integrity and decrease mitochondrial depolarization in oxidative-stress thereby decreasing concomitant caspase-3 activation [31, 33].

Clinical implications

As these findings are novel, transitioning these results from the bench to the bedside will not happen quickly. Much work remains to both replicate these findings in higher species and to explore other m_{KATP} agonists used subsequent to hemorrhagic shock. However, if these findings can be replicated in higher species, the ability to promote organ protection *after the fact* following hemorrhagic injury has substantial clinical implications. This investigation found that DZ could be safely administered at 3.2 mg/kg IV after hemorrhagic shock and at two time points either increased HSP expression or decreased caspase-3 activation. DZ could provide civilian first responders and military medics a tool to

promote survival following trauma as they could administer DZ intravenously with a dosing that does not promote hypotension.

Additionally, this study found that mixing DZ into 0.9% NS resuscitation fluid decreased caspase-3 activation. Experiments, using DZ in other fluid types or using other mK_{ATP} agonists in 0.9% may be useful in determining the benefit of augmenting already clinically used fluids with pharmaceutical agents that promote a prosurvival phenotype. If future studies found these interventions protective, trauma centers may consider using mK_{ATP} agonists during trauma resuscitation.

Future studies

Research into postconditioning resuscitation for hemorrhagic shock is in its infancy. This investigation has created a wide range of questions that need further exploration. These findings were a result of IV injections of DZ. While promising it does have some limitations in trauma care. It is not always possible to quickly obtain intravenous access following hemorrhagic trauma. Thus, IM injections would be useful in this setting. Replicating this study with IM injection of DZ would be prudent. On the battlefield it is suggested that resuscitation should be conducted with small volume 7.5% hypertonic saline. In this study, DZ mixed into the 0.9% NS decreased caspase-3 activity. Replicating this study using hypertonic saline and the IV or IM injection of DZ would be relevant for military medicine.

This investigation looks at molecular effects 24 hours following injury. Neither behavioral assays nor extended molecular tests were conducted. Chapter 3 results suggested that shock affects several areas within subregions of the cerebral cortex, hippocampus, thalamus, hypothalamus, and amygdala. The model used in this investigation lends itself to behavioral investigation as there is no extremity injury and motor or sensory deficits could be evaluated to see if DZ is of benefit given subsequent to hemorrhagic shock. In addition to assessment of functional outcomes over time, molecular processes can vary. This study looked at protein expression and caspase-3 activity 24 hours after injury. Future investigations of 3-7 days post injury are necessary as cell death may not be evident within the first 24 hours.

In summary, this research provides a glimpse of the possibility to invoke postconditioning following hemorrhagic shock. Bench science research, as proposed here, considers and explores new approaches to trauma resuscitation and holds promise for developing new strategies for post-traumatic care. Although transition of these results to the bedside is a long journey, these results suggest that trauma postconditioning could reduce brain injury and possibly a decrease in morbidity and mortality.

Figure 1. Post-hemorrhagic pre-resuscitation conditioning using diazoxide increases HSP25 and HSP70 in the cerebral cortex and hippocampus.

Post-hemorrhagic pre-resuscitation conditioning with intravenous diazoxide greatly increases HSP25 and HSP70 in the ipsilateral (to occlusion) rat cerebral cortex and hippocampus in a model of hemorrhagic shock and unilateral carotid artery occlusion.

Figure 1. Post-hemorrhagic pre-resuscitation conditioning using diazoxide increases HSP25 and HSP70 in the cerebral cortex and hippocampus.

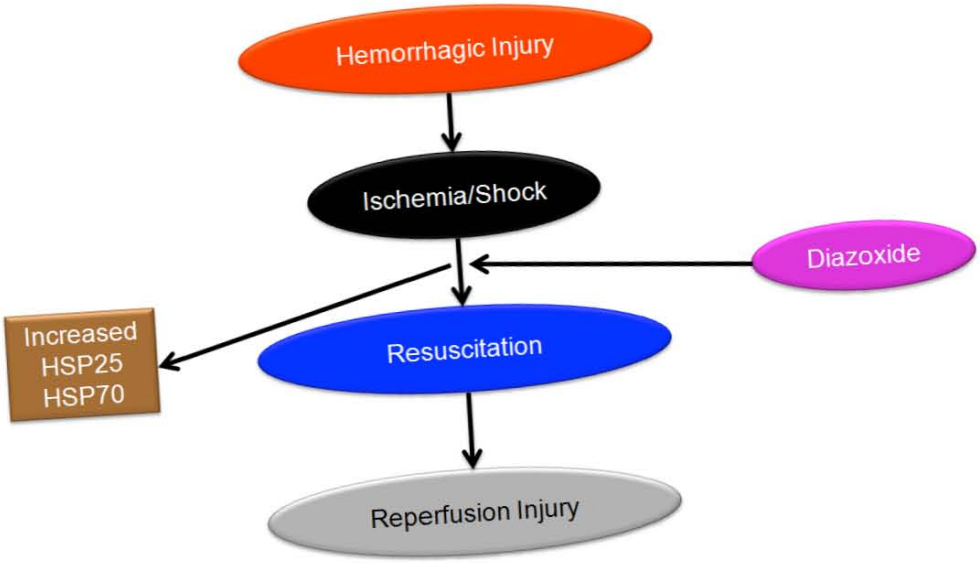
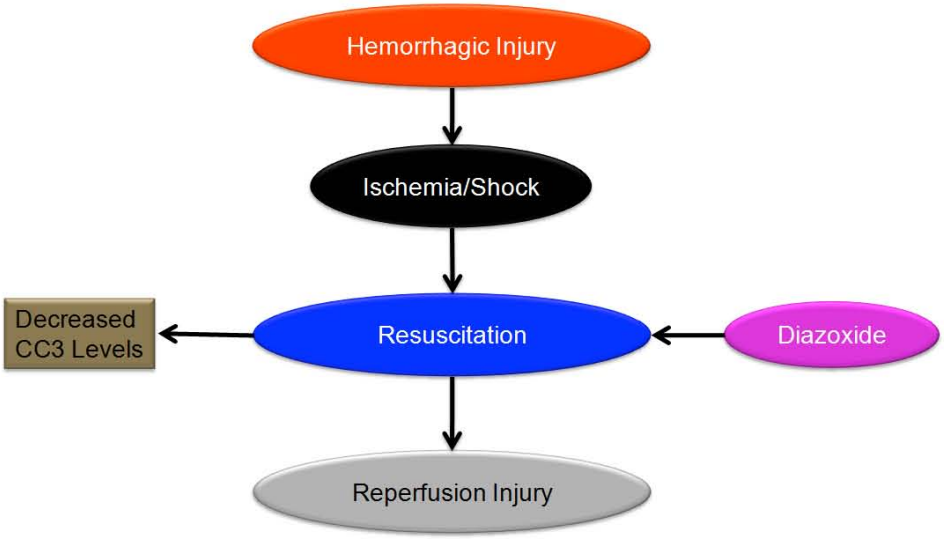


Figure 2. Resuscitation with diazoxide decreases hemorrhagic shock-induced brain levels of cleaved caspase-3.

Intravenous diazoxide when given concomitantly with resuscitation (mixed within the initial resuscitation volume) significantly decreased cleaved caspase-3 activity bilaterally in the hippocampus and piriform cortex.

Figure 2. Resuscitation with diazoxide decreases hemorrhagic shock-induced brain levels of cleaved caspase-3.



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Appendix: Physiological Response to Shock

Figure 1: Mean arterial pressure (MAP) after hemorrhagic shock.

An overall significant difference in MAP between the non-shocked (UCAO, UCAO VEH, UCAO DZ) and shocked (HSCH, DZ40, DZ60, DZ80) groups was evident during the shock period before resuscitation (significant differences indicated by the black bar above the abscissa). At Time 0, there was no difference in MAP between the Groups. From 5 min to 65 min, the non-shocked Groups MAPs were significantly greater than the shocked Groups with the exception of the 5 min time point when the UCAO VEH Group MAP was greater than the MAP of the UCAO Group. Finally, the MAPs at Time 0 were significantly greater than at the 5 min to 75 min time measures for the shocked Groups. These results should be interpreted with caution, however, since the ANOVA failed the normality test and the equal variance test. Conventional transformations of the data did not alter normality or variance heterogeneity.

Figure 1: Mean MAP after hemorrhagic shock

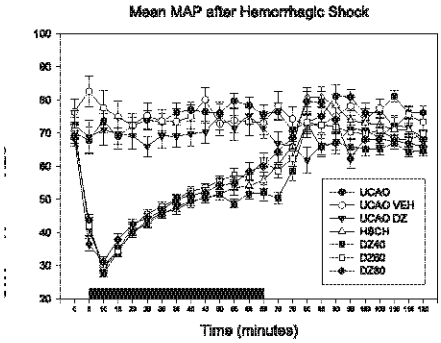

Figure 2: Mean heart rate (HR) after hemorrhagic shock.

At Time 0, all of the Groups that sustained UCAO (with or without subsequent hemorrhagic shock) exhibited lower HRs than the Control Group, but this was not a significant difference. From 5-35 min, there were some group differences where the HR for the Control Group was greater than the HSCH, DZ60 and DZ80 Groups, and by 10 min all of the shocked Groups (HSCH and all DZ-treated Groups) were less than the Control Group. Over time there was a recovery of HR for all groups that sustained UCAO or shock, and from 40 min after the study began to its conclusion the HRs were not different between Groups. There was an observed trend, however, in physiological response to UCAO and shock where, compared to no UCAO, carotid occlusion reduced HR, and shock further reduced HR. To further assess the impact of UCAO and shock, the data were re-analyzed to directly compare the Control animals, the UCAO Groups (UCAO, UCAO VEH, UCAO DZ) and the Groups that received shock (HSCH, DZ40, DZ60, DZ80). As shown in the figure below, at Time 0 (i.e., when all animals except the Group UCAO), there was a significant difference between the UCAO Group and all other Groups, which exhibited a decrease in HR (indicated by *). From 5-25 min, there was a further difference between the Groups where the average HR for the animals in the Control Group was significantly greater than both the UCAO and Shocked Groups (indicated by black bar on abscissa). At 30-50 min, in turn, the UCAO Group HR was greater than in the Shocked Group (i.e., Control HR > UCAO HR > Shocked Group HR, indicated by hatched bar on abscissa), while from 55-80 min into the study the Groups did not.

Figure 2: Mean Heart Rate after Hemorrhagic Shock

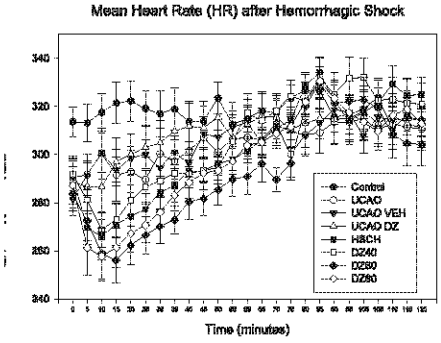

Figure 3: Mean heart rate (HR) after hemorrhagic shock across the three major treatment conditions.

To further assess the impact of UCAO and shock, the data were re-analyzed to directly compare the Control animals, the UCAO Groups (UCAO, UCAO VEH, UCAO DZ) and the Groups that received shock (HSCH, DZ40, DZ60, DZ80). As shown in the figure below, at Time 0, there was a significant difference between the Control group and the UCAO and Shocked Groups, which exhibited a decrease in HR (indicated by *). From 5-25 min, there was a further difference between the Groups where the average HR for the animals in the Control Group was significantly greater than both the UCAO and Shocked Groups (indicated by black bar on abscissa), and the UCAO Group had significantly higher HRs than the Shocked Groups (i.e., Control HR > UCAO HR > Shocked Group HR). At Times 30-50 min, in turn, the Control and UCAO Groups HR was greater than in the Shocked Group (indicated by hatched bar on abscissa), while from 55-80 min into the study the Groups did not differ.

Figure 3: Mean Heart Rate after Hemorrhagic Shock

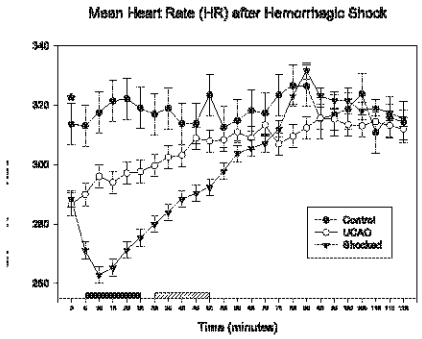

Table 1. Summary: Change over time of Arterial Blood Gas (ABG) Data.

ABG data was assessed immediately after hemorrhage, 1 hour after hemorrhage and at the end of full resuscitation. Probability values after a Krusal-Wallis ANOVA are listed, which compare blood values the UCAO, UCAO VEH, UCAO DZ, HSCH, DZ40, DZ60, and DZ60 Groups. In some cases (indicated by n.s. after the *p*-value), the overall ANOVA was significant but differences between individuals Groups was not significant. There was a significant decrease in pH when comparing the UCAO group to all shocked groups following full resuscitation. Immediately following hemorrhage and one hour following hemorrhage, lactate levels in all shocked groups were elevated compared to the UCAO group. BEecf (extracellular fluid) was significantly decreased in the HSCH, DZ40, and DZ80 Groups following resuscitation when compared to the UCAO Group. Bicarbonate ion at no time point was significantly different, however p=0.061 at the end of resuscitation.

Table 1. Summary: Change over time of Arterial Blood Gas Data.

Summary of Physiological Data*			
Measure	**15 min (immediately at end of hemorrhage)**	**75 min (60 minutes after hemorrhage)**	**120 min (after full resuscitation)**
DELTA			
pH	p=0.003; n.s.	p=0.007; n.s.	p<0.001; UCAO > all shock groups
Lactate	p<0.001; UCAO < all shock groups	p<0.001; UCAO < all shock groups	p=0.336
Glucose	p<0.001; UCAO < HSCH, DZ80 groups	p<0.003; UCAO < DZ40, DZ60, DZ80	p=0.101
BEecf	p=0.077	p=0.160	p=0.007; UCAO> HSCH, DZ40, DZ80
HCO_3	p=0.161	p=0.313	p=0.061

*KW ANOVA used to compare Groups at each separate time point, *post hoc* Dunn's Test compares Groups to the UCAO Group.

Table 2. Raw arterial blood gas (ABG) data for pH, glucose, and lactate measurements.

The data represents the raw data from the arterial blood gases taken from the rats. In this table, the times (Time 2, Time 3, and Time 4) refer to the 15 minute, 75 minute, and 120 minute point in the experiment. The values given are the difference from the initial baseline value taken 5 minutes into the procedure (Time 1).

Table 2. Raw arterial blood gas (ABG) data for pH, glucose, and lactate measurements.

pH Data						Glucose Data						Lactate Data					
Time 2						Time 2						Time 2					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	14	0	0.0106	0.0235	6.29E-03	UCAO	9	0	-9.556	21.237	7.079	UCAO	11	0	-0.364	0.391	0.118
UCAO VEH	6	0	0.019	0.0326	0.0133	UCAO VEH	7	0	-35.857	38.667	14.615	UCAO VEH	8	0	-0.175	0.231	0.0818
UCAO DZ	7	0	8.71E-03	0.0407	0.0154	UCAO DZ	5	0	-20	53.193	23.789	UCAO DZ	8	0	0.15	0.359	0.127
HSCH	13	0	0.0125	0.0532	0.0148	HSCH	9	0	37.333	31.611	10.537	HSCH	12	0	2.308	1.28	0.37
DZ40	17	0	-0.0267	0.0392	9.51E-03	DZ40	13	0	32.846	44.164	12.249	DZ40	15	0	3.153	1.282	0.331
DZ60	14	0	0.0517	0.0452	0.0121	DZ60	9	0	31	52.292	17.431	DZ60	10	0	3.59	2.155	0.682
DZ80	15	0	-4.67E-03	0.0584	0.0151	DZ80	10	0	56.2	21.467	6.789	DZ80	14	0	3.336	0.923	0.247
Time 3						Time 3						Time 3					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	14	0	0.0198	0.0463	0.0124	UCAO	9	0	-25.889	13.448	4.483	UCAO	11	0	-0.555	0.731	0.22
UCAO VEH	6	0	0.0773	0.119	0.0484	UCAO VEH	7	0	-13.714	46.707	17.654	UCAO VEH	8	0	-0.275	0.544	0.192
UCAO DZ	7	0	0.0269	0.0272	0.0103	UCAO DZ	5	0	-31.4	41.855	18.718	UCAO DZ	8	0	0.025	0.625	0.221
HSCH	13	0	-0.0137	0.074	0.0205	HSCH	9	0	19.667	115.447	38.482	HSCH	12	0	1.883	1.78	0.514
DZ40	17	0	-0.0385	0.0624	0.0151	DZ40	13	0	63.385	79.184	21.962	DZ40	15	0	2.44	2.568	0.663
DZ60	14	0	0.0114	0.0613	0.0164	DZ60	9	0	62.778	93.621	31.207	DZ60	10	0	2.05	2.544	0.804
DZ80	15	0	-0.0185	0.057	0.0147	DZ80	10	0	48.3	52.57	16.624	DZ80	14	0	1.471	1.219	0.326
Time 4						Time 4						Time 4					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	14	0	0.0204	0.0463	0.0124	UCAO	9	0	-25.889	17.885	5.962	UCAO	11	0	-0.545	0.849	0.256
UCAO VEH	6	0	0.0227	0.0431	0.0176	UCAO VEH	7	0	-21	66.858	25.27	UCAO VEH	8	0	-0.35	0.404	0.143
UCAO DZ	7	0	0.0216	0.0288	0.0109	UCAO DZ	5	0	0.6	31.021	13.873	UCAO DZ	8	0	0.05	0.659	0.233
HSCH	13	0	-0.0413	0.0768	0.0213	HSCH	9	0	-34.556	75.667	25.222	HSCH	12	0	-0.1	1.24	0.358
DZ40	17	0	-0.0648	0.08	0.0194	DZ40	13	0	-10.923	39.959	11.083	DZ40	15	0	-0.113	1.867	0.482
DZ60	14	0	-0.0427	0.0576	0.0154	DZ60	9	0	19.111	47.343	15.781	DZ60	10	0	-0.15	1.061	0.335
DZ80	15	0	-0.077	0.0779	0.0201	DZ80	10	0	3.6	61.787	19.539	DZ80	14	0	0.0357	0.855	0.229

Table 3. Raw arterial blood gas (ABG) data for base excess (BEcf) and bicarbonate (HCO3) measurements.

This table reflects the actual value for base excess and bicarbonate ion at each sample extraction at 5 minutes, 15 minutes, 75 minutes, and 120 minutes of the procedure (Time 1, Time 2, Time 3, and Time 4 respectively).

Table 3. Raw arterial blood gas (ABG) data for BEcf and HCO3 measurements.

BEcf Data						HCO3 Data					
Time 1						Time 1					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	12	0	9.508	4.39	1.267	UCAO	12	0	32.833	3.893	1.124
UCAO Veh	6	0	8.583	2.832	1.156	UCAO Veh	6	0	31.95	2.803	1.144
UCAO DZ	5	0	4.1	6.102	2.729	UCAO DZ	5	0	28.74	5.519	2.468
HSCH	11	0	12.382	3.185	0.96	HSCH	11	0	35.764	2.685	0.81
DZ40	16	0	11.612	2.617	0.654	DZ40	16	0	34.925	2.552	0.638
DZ60	11	0	11.545	3.425	1.033	DZ60	11	0	35.573	3.843	1.159
DZ80	15	0	12.267	2.802	0.724	DZ80	15	0	35.713	2.683	0.693
Time 2						Time 2					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	12	0	8.383	4.444	1.283	UCAO	12	0	31.5	4.038	1.166
UCAO Veh	6	0	8.333	2.071	0.846	UCAO Veh	6	0	31.2	2.072	0.846
UCAO DZ	5	0	4.44	8.214	3.674	UCAO DZ	5	0	28.98	6.928	3.098
HSCH	11	0	5.673	3.397	1.024	HSCH	11	0	28.991	3.411	1.029
DZ40	16	0	4.838	2.227	0.557	DZ40	16	0	28.488	2.287	0.572
DZ60	11	0	5.318	3.463	1.044	DZ60	11	0	28.427	3.14	0.947
DZ80	15	0	5.847	1.975	0.51	DZ80	15	0	29.313	1.696	0.438
Time 3						Time 3					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	12	0	8.283	3.413	0.985	UCAO	12	0	31.1	3.561	1.028
UCAO Veh	6	0	7.917	4.174	1.704	UCAO Veh	6	0	30.717	3.853	1.573
UCAO DZ	5	0	2.72	9.047	4.046	UCAO DZ	5	0	26.8	8.038	3.595
HSCH	11	0	5.682	2.977	0.898	HSCH	11	0	29.291	2.566	0.774
DZ40	16	0	4.244	4.035	1.009	DZ40	16	0	28.087	3.677	0.919
DZ60	11	0	5.273	3.561	1.074	DZ60	11	0	28.818	3.057	0.922
DZ80	15	0	6.167	3.621	0.935	DZ80	15	0	29.74	3.527	0.911
Time 4						Time 4					
Group Name	N	Missing	Mean	Std Dev	SEM	Group Name	N	Missing	Mean	Std Dev	SEM
UCAO	12	0	7.133	3.9	1.126	UCAO	12	0	29.792	3.959	1.143
UCAO Veh	6	0	8.45	3.583	1.463	UCAO Veh	6	0	31.25	3.37	1.376
UCAO DZ	5	0	3.5	7.583	3.391	UCAO DZ	5	0	27.7	6.443	2.881
HSCH	11	0	2.309	2.151	0.649	HSCH	11	0	26.418	2.017	0.608
DZ40	16	0	2.194	3.926	0.981	DZ40	16	0	26.45	3.356	0.839
DZ60	11	0	3.045	4.223	1.273	DZ60	11	0	27.555	4.135	1.247
DZ80	15	0	2.247	4.656	1.202	DZ80	15	0	26.867	4.163	1.075

Table 4: Fluorojade (FJC) Observations

Observations were made under a 50x objective for the presence or absence of a FJC staining and categorized as “1” for present or “0” for absent and the group results were tallied.

Table 4: Fluorojade (FJC) Observations

FJC Staining	RCC	RHIP	LCC	LHIP
Control (n=5)	0	0	0	0
Sham (n=5)	0	0	0	0
UCAO (n=5)	0	0	0	0
UCAO VEH (n=7)	0	1	0	0
UCAO DZ (n=5)	0	1	0	0
HSCH (n=9)	2	5	0	0
DZ40 (n=7)	4	3	0	0
DZ60 (n=6)	0	4	0	0
DZ80 (n=6)	0	2	0	0
Totals (n=55)	6	16	0	0

Figure 4. GFAP Measurements in the Left Hemisphere.

Images of selected regions of the left cortex and hippocampus were obtained using a 20x objective. Using ImageJ software, images were converted to a binary image and percent area was calculated and recorded. The bar graphs represent the average of each group with error bars noting the standard deviation.

Figure 4. GFAP Measurements in the Left Hemisphere

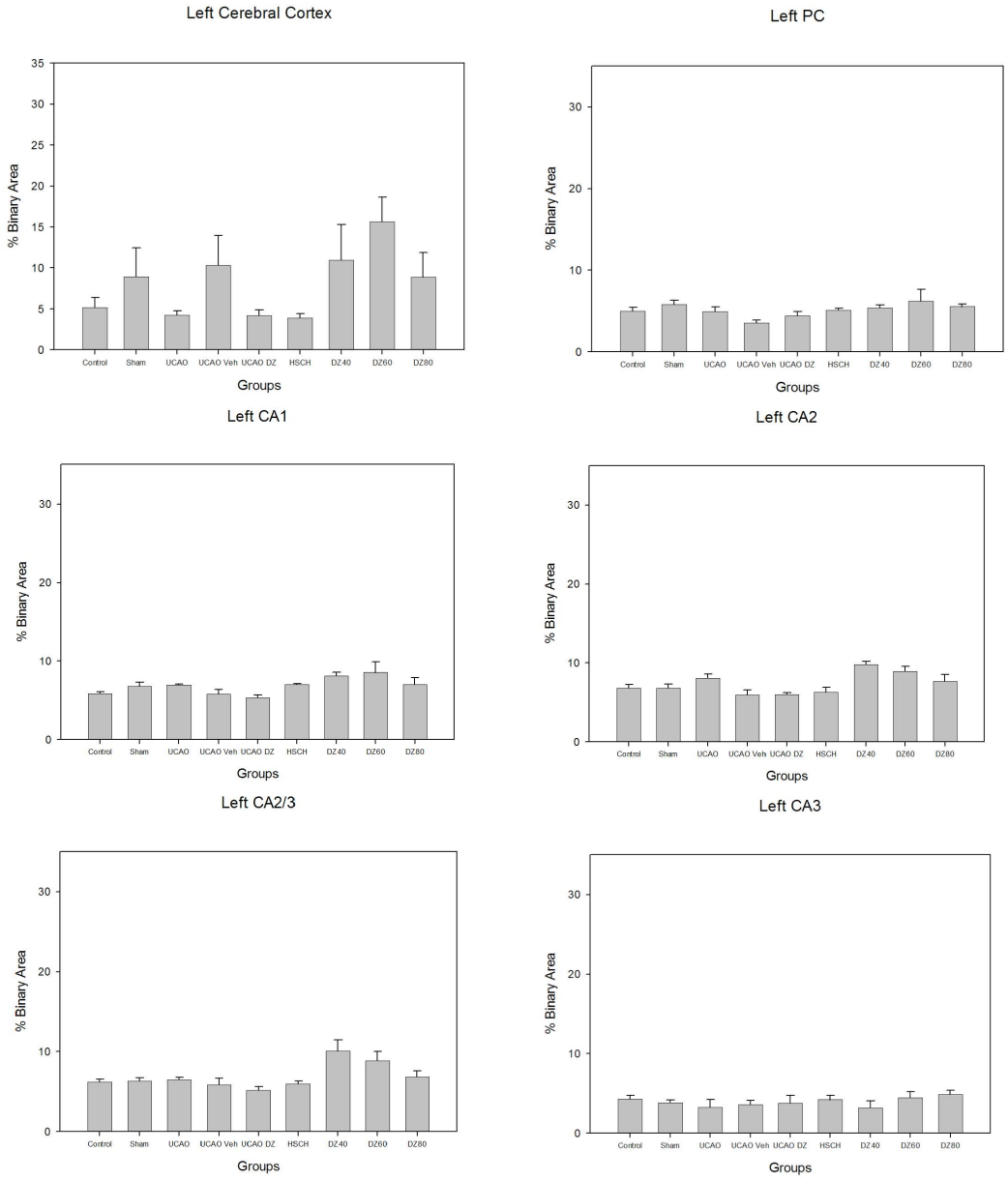

Figure 5. GFAP Measurements in the Right Hemisphere

Images of selected regions of the right cortex and hippocampus were obtained using a 20x objective. Using ImageJ software, images were converted to a binary image and percent area was calculated and recorded. The bar graphs represent the average of each group with error bars noting the standard deviation.

Figure 5. GFAP Measurements in the Right Hemisphere